COMPARISON OF THE LEVELS OF SELECTED SPECIFIC ANTIBODIES IN THE IgG OF COLOSTRUM, MILK AND SERUM IN DAIRY COWS (*BOS TAURUS*)

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ABSTRACT

In cattle (*Bos taurus*), the immunologically naïve calf receives immune factors, growth factors and nutrients from the dam via the colostrum. Colostral antibodies are primarily serum-derived and provide the calf with the broad-spectrum systemic immunity of the mother. There are a variety of commercially available products used to supplement or replace maternal colostrum. In addition to utilizing colostrum as the IgG source, there is an interest in using IgG from milk and serum as ingredients in colostrum replacement products. Immunoglobulins in milk are primarily derived from udder-localized plasma cells, which migrate from the intestinal mucosa at parturition and during lactation. We hypothesized that milk IgG would have lower levels of antibodies to systemic pathogens compared to colostrum and/or serum derived IgG however might contain higher levels of antibodies to mucosal and udder associated agents. We sampled serum (1-2hr post partum), colostrum (1-2hr post partum) and milk (day 5 post partum) from 24 post parturient dairy cows and heifers, measured total IgG (H+L) using a radial immunodiffusion assay (RID), and determined specific antibody units per gram of IgG for a variety of respiratory, gut and udder-associated pathogens via indirect Enzyme-Linked Immunosorbant Assay (ELISA) (ie., BRSV, BHV-1, PI3, *Streptococcus uberis*, *Staphylococcus aureus*., *E. coli* F5 (K99), rotavirus and bovine coronavirus). We performed additional ELISAs to determine antigen specific values for IgG1 and IgG2 antibody subclasses for BRSV, rotavirus and *S. uberis*. Wilcoxon signed-rank tests with conservative Bonferroni correction, showed that antigen specific antibodies to BRSV (IgG H+L), PI3 (IgG H+L), *E. coli* F5 (K99) (IgG H+L), *S. aureus* (IgG H+L), rotavirus (IgG H+L and IgG1) and BCV (IgG H+L) are higher (P<.017) in the IgG in colostrum than the IgG in milk. In comparison to the serum, colostrum IgG is higher (P<.017), in BRSV (IgG H+L and IgG1), BHV1 (IgG H+L), PI3 (IgG H+L), *E. coli* F5 (K99) (IgG H+L), *S. uberis* (IgG H+L), and rotavirus (IgG1) antibodies. Colostrum IgG also contains more specific antibody to *S. uberis* (IgG1) and BCV (H+L) than serum IgG. However, milk IgG contains more specific antibody to BRSV (IgG H+L and IgG1), BHV1 (IgG H+L), PI3 (IgG H+L), and rotavirus (IgG1) when compared to serum IgG (P<.017). Overall, colostrum derived IgG delivers more specific antibodies to most endemic pathogens compared to the IgG found in milk or serum. However, milk IgG, like that of colostrum, has higher amounts of specific IgG1 and delivers a similar spectrum of specific antibodies and thus may be a superior Ig source for the newborn calf when compared to serum IgG.

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DEDICATION

This thesis is dedicated to my parents. I wish you could have been here.

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LIST OF ABBREVIATIONS

AEA – Apparent Efficiency of Absorption

ABTS - 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid

BRD – Bovine Respiratory Disease

BRSV – Bovine Respiratory Syncytial Virus

CR – Colostrum Replacer

CS- Colostrum Supplement

E. coli – *Escherichia coli*

FcRn – Neonatal Fc Receptor

FPTI – Failure of Passive Transfer of Immunity

GALT – Gut-Associated Lymphoid Cells

GI – Gastrointestinal

H+L – Heavy and Light Chain

Ig – Immunoglobulin

IgA – Immunoglobulin A

IGF1 – Insulin-Like Growth Factor 1

IgG – Immunoglobulin G

IgG1 – Immunoglobulin G1

IgG2 – Immunoglobulin G2

IgM – Immunoglobulin M

IL-6 – Interleukin 6

MC – Maternal Colostrum

OD – Optical Density

PI3 – Parainfluenza3

pIGR – Polymeric Immunoglobulin Receptor

S. aureus – *Staphylococcus aureus*

S. uberis – *Streptococcus uberis*

TNF-a – Tumor Necrosis Factor alpha

TP – Serum Total Protein

USDA – United States Department of Agriculture

GENERAL INTRODUCTION

The beef and dairy industries are of significance to the Canadian agricultural economy, netting respectively $9.4 and $6.99 billion in farm cash receipts in 2019 alone. However, studies report calf morbidity in dairy herds as high as 34-35% (Urie et al., 2018; Waltner-Toews et al., 1986), and in the United States, pre-weaned dairy calves have an average mortality rate of between 5-11% (Beam et al., 2009; Urie et al., 2018; *USDA APHIS | NAHMS Dairy Studies*, 2007, 2014). These elevated rates of morbidity and mortality ultimately translate to reduced productivity, economic losses due to costs of therapies, reduced weight gain, and deaths (Rossini, 2004; Sivula et al., 1996; Stanton et al., 2012). The most common causes of morbidity and mortality in calves are neonatal calf diarrhea and bovine respiratory disease (BRD) (Donovan et al., 1998; Van Donkersgoed et al., 1993; Wells et al., 1996). Both are multifactorial diseases, classified as such because while the causative agents must be present for disease development, their presence alone is not sufficient to cause disease. In fact, most of these pathogens are environmentally ubiquitous and thus exposure for neonatal calves in livestock operations is guaranteed.

In ruminants, the young are born almost agammaglobulinemic and the passive transfer of immunoglobulins from maternal colostrum is critical to the survival of the neonate in the first weeks until its own immune responses are protective (Gullickson et al., 1942). Failure of passive transfer of maternal immunity is the primary factor contributing to the mortality of pre-weaned calves and is, therefore, a major economic consideration for livestock producers (Windeyer et al., 2014). Colostrum replacer products (CR) provide a practical way to improve passive transfer in calves, allowing producers to feed a known mass of IgG. However, the results of colostrum replacer trials have been inconsistent, with many products failing to provide the serum levels of IgG considered the minimum for successful passive transfer (reviewed in, Cabral et al., 2013; reviewed in, Godden & James, 2020). Additionally, depending upon the CR product, feeding the same IgG mass in maternal colostrum and CR formulations may not provide the same rates of passive transfer (Cabral et al., 2013; reviewed in, Godden & James, 2020).

CR products may be derived from colostrum, milk whey, serum or a combination thereof. Variable success of CR products in achieving adequate passive transfer IgG levels may be partially due to differences in apparent efficiency of absorption (AEA) of immunoglobulin from colostrum, whey and serum-derived products compared to natural colostrum (reviewed in, Cabral et al., 2013).

There are a number of other potential reasons for poor performing CR products. Colostrum must go through a great deal of processing, including heat treatment and spray drying for use as a safe and shelf-stable product and that processing must be closely monitored to prevent damage to proteins and other components (Campbell et al., 2007; Thornsberry & Wood, 2011). Manufacturer instructions for reconstituting, feeding and feeding times must be closely observed. There is some evidence that high levels of other proteins in the colostrum product can competitively bind protein-binding sites and channels, inhibiting the non-selective uptake of immunoglobulins and other immune factors (Besser & Osborn, 1993). Colostrum also likely contains factors that increase the rate of gut closure, effectively decreasing the window for passive transfer (Besser & Osborn, 1993).

Maternal colostrum is the most obvious source of Igs for CR products. Immunoglobulins found in colostrum are selectively transferred from the serum into the udder by receptor-mediated processes in the weeks leading up to parturition. These immunoglobulins provide systemic immunity and, through resecretion, mucosal immunity that is both broad-spectrum and environmentally relevant to the neonate (reviewed in, Hurley, 2003). While the ideal choice of immunoglobulin for CR formulations is therefore assumed to be colostrum, high quality maternal colostrum (MC) is expensive, in short supply, and even MC-derived CR products do not consistently perform as well as high quality maternal colostrum (reviewed in, Cabral et al., 2013).

Serum is a potential source of immunoglobulins in CR products. Large volumes of serum are available as a by-product of the slaughterhouse industry. Serum contains a broad range of immunoglobulins, with the IgG1 and IgG2 subclasses of IgG represented equally. To date, studies investigating the performance of serum-derived CR products are mixed. If fed containing high enough IgG mass, serum-based products can provide neonates with adequate serum immunoglobulin levels to meet the standards for successful passive transfer of immunity (Arthington et al., 2000). However, the variable success of serum based CR products indicates that these products are not without functional and absorptive issues (reviewed in, Cabral et al., 2013; reviewed in, Godden & James, 2020). Most pertinent is that serum is composed of an equal ratio of IgG1 and IgG2 subclasses, while maternal colostrum contains primarily the IgG1 subclass and only IgG1 has been shown to have the capacity for re-secretion and thus protection at mucosal surfaces (Besser, McGuire, et al., 1988; Ellis et al., 2018). Additionally, serum products appear to

have reduced efficiency of absorption of immunoglobulins (Campbell et al., 2007; reviewed in, Godden et al., 2019; Thornsberry & Wood, 2011).

Antibodies in milk, in contrast to both colostrum and serum, are assumed to be primarily derived locally in the udder, and potentially from cells that migrate from the intestinal mucosa during lactation, possibly providing the newborn calf preferentially with antibodies to agents associated with mucosal surfaces; particularly pathogens responsible for neonatal calf diarrhea (Besser, McGuire, et al., 1988; Butler et al., 1972, 1986; Sordillo & Nickerson, 1988). Dairy cows produce milk in excess of the nutritional needs of the calf, and the excess is regularly harvested and processed in large volumes in the dairy and cheese industries, leaving an excess of Ig-rich milk whey as a by-product. The spectrum of milk antibody types (IgG, IgA, IgM) and subclasses is similar to that of colostrum with a high proportion comprised of the IgG1 subclass (reviewed in, Larson, 1992; reviewed in, Tizard, 2018). Though the concentration of immunoglobulin in the milk is quite low, it can be concentrated. A number of naturally occurring enzymes and compounds including lactoferrin, lactoperoxidase and lysozyme are currently harvested from milk for the health and cosmetics industries. Additionally, hyperimmune milk from cows vaccinated to increase targeted antibody responses to rotavirus and cryptosporidium have been used to treat these infections in people (Mitra et al., 1995; Okhuysen et al., 1998). The similar IgG subclass ratio and the potential for greater proportions of some important specific immunoglobulins compared even to colostrum, suggests that milk IgG is an appropriate ingredient in colostrum replacer for calves. Milk derived immunoglobulin has been used in some countries including the USA for many decades as an ingredient in CR products (Mee et al., 1996), however its efficacy has not been studied or compared to other sources.

Colostrum management programs on farms are a vital part of maintaining calf health and improving economic efficiencies. CR products can provide convenient, consistent nutrition and immune factors, and allow cattle operations to break the cycle of transmission of some common bovine pathogens. Currently, there are no studies addressing concerns that the specificity of immunoglobulins in milk whey or serum may differ from those of colostrum and the potential for those differences to impact the efficiency of the immunoglobulin of the IgG in colostrum replacement formulas. A more complete understanding of the antibody specificity of colostrum,

serum and milk could influence their use and potentially lead to improved colostrum management tools and improved calf health and performance outcomes.

1. LITERATURE REVIEW

1.1 Passive Immunity

Passive immunity is defined as the transfer of specific antibodies or immunoglobulins from one individual to another to provide short-term protection against infection or disease. First demonstrated experimentally over 100 years ago to protect rabbits from lethal doses of cobra venom, passive immunization has since been utilized extensively to treat and prevent diseases in both animals and humans (Calmette, 1896; Eibl, 2008; Hsu & Safdar, 2011). The passive immunity provided through the transfer of immunoglobulins from mother to progeny (passive transfer of maternal immunity) offers immediate protection via environmentally relevant antibodies, developed in response to the antigenic challenges faced by the dam in the weeks leading up to parturition. The mechanism through which antibodies are passed to the young of a given species is variable. In mammals, protective immunoglobulin may be exchanged via the placenta, through the mammary gland secretions, or a combination of the two (reviewed in, Butler et al., 2015; Butler & Kehrli, 2005; Hurley, 2003). In contrast to mice and humans, that receive immunoglobulins *in utero*, many domestic animals receive most or all of their passive immunity via the placenta. In cattle (*Bos taurus*), and other ruminants, the placental structure completely prevents *in utero* transmission of immunoglobulin (Arthur et al., 1996; reviewed in, Butler et al., 2015; Hurley & Theil, 2011; Palmeira et al., 2012).

Ruminants, therefore, are born almost agammaglobulinemic and are highly susceptible to infectious disease. Newborn ruminants receive all systemic passive immunity through the first post partum mammary secretion, the colostrum (Senger, 1997). Maternal Igs in the colostrum are ingested and absorbed across the lamina propria of the small intestine and move into the bloodstream during the first 24 hours following birth (Hurley & Theil, 2011; Palmeira et. al., 2012).

Passive immunization through the oral administration of exogenous immunoglobulins may also supplement local mucosal immunity in individuals incapable of systemic absorption of immunoglobulin (Besser, Gay, et al., 1988; Saif et al., 1983). Passive immunization via antibodies in milk diets has proven to be a particularly effective strategy for the protection of young livestock animals in the face of common enteric pathogen challenges, blocking the attachment of pathogens and promoting their excretion (Arthington et al., 2002; Berge et al., 2009, 2009; Jones et al., 1988;

Nagy, 1980; Parreño et al., 2004, 2010; Rousic et al., 2000; Saif & Fernandez, 1996).

In calves, studies with radio-labelled immunoglobulins show no intestinal absorption of IgG1 from the milk, demonstrating endogenous production of IgG1 starting as early as 36h after birth (Devery et al., 1979). However, innate antibody production appears to be tied to passive transfer level. In calves with high serum IgG from passive transfer, IgG production begins at 4 weeks, while hypogammaglobulinemic calves with low passive transfer begin producing antibodies at 7 days of age (Logan et al., 1974).

1.1.1 Maternal Immunoglobulin

Maternal immunoglobulin, transferred from dam to offspring, can have lifesaving, disease-sparing effects for a variety of neonatal infections. The proportions of immunoglobulin classes (IgG, IgM, and IgA) in the colostrum and milk are variable among species. In cattle, the neonate receives passive immunity via maternal antibodies in both the colostrum and milk. IgM and IgA occur at relatively low concentration in ruminant colostrum and milk. IgG is the primary Ig in serum, and in cattle is also the primary Ig in both colostrum and milk. These antibodies are responsible for identifying foreign pathogens, which are then recognized by macrophages and neutrophils enabling their eventual destruction by phagocytosis or through the activation of the complement cascade.

In cattle there are several subclasses of IgG. IgG1 and IgG2 are the major immunoglobulins, occurring in similar proportions in serum. In contrast, IgG1 comprises the primary immunoglobulin in bovine colostrum and milk (Barrington, Besser, Davis, et al., 1997). IgG1 and 2 differ structurally in the hinge sites and CH2 domains involved in binding to Fc receptors. IgG1 is the major antibody of secondary immune responses, primarily recognizing bacterial toxins and viral proteins, while IgG2 primarily recognizes the polysaccharide coatings of bacteria such as *Streptococcus pneumonidae* and *Haemophilus influenzae* (Vidarsson et al., 2014). Only IgG1 can be transferred, through specific receptors, from the circulation to the nasal and intestinal mucosa for additional protection at major sites of pathogenic challenge.

1.1.2 Colostrum and Passive Protection of the Newborn

Colostrum is the nutrient and immunoglobulin-rich first secretion of the mammary gland of mammals following parturition. Its purpose is to provide energy and immediate immunity to the neonate and thus it has a significantly different nutrient and immunological profile from milk.

Colostrum production and composition are influenced by breed genetics (Barrington et al., 2001; Norman et al., 1981). It is assembled during the five-week dry/non-milking period leading up to calving when the upregulation of lactogenic hormones signals the accumulation of lacteal secretions and serum components in the udder. This accumulation ceases at parturition most likely due to increases in prolactin concentration at the onset of lactation (Barrington et al., 2001; reviewed in, Foley & Otterby, 1978; Larson et al., 1980; Rincheval-Arnold et al., 2002). Colostrum is harvested as the udder is suckled or milked in the first 1 to 3 days postpartum. The components, including the immunoglobulin, are rapidly depleted by milking or suckling and/or are diluted in concentration by the influx of milk, produced starting at parturition.

While colostrum and milk are both udder secretions that provide nourishment to support the growing calf, the composition of each varies substantially. Both contain protein, fat, and micronutrients, however, colostrum has double the solids, twice as much fat, and four times the total protein compared to milk (reviewed in, Foley & Otterby, 1978; Playford & Weiser, 2021). Additionally, colostrum is richer in oligosaccharides, growth factors, antimicrobial compounds, and immune components than the milk (Davis & Drackley, 1998; Gopal & Gill, 2000; Quigley & Drewry, 1998). Fat, present in higher concentration in colostrum, provides energy for thermogenesis in the initial days following birth (Davis & Drackley, 1998). Energy and vital micronutrients work synergistically to initiate metabolism and stimulate the development of the digestive system in the neonate (Davis & Drackley, 1998). As well as immunoglobulins, colostrum provides other important immune factors and non-specific anti-microbial factors such as insulin-like growth factor (IGF1), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-a), and lactoferrin which provide non-specific protection (Reiter et al., 1975; Wheeler et al., 2007). Lactoferrin in colostrum is bacteriostatic, binding iron and transporting it to intra-cellular sites, preventing uptake by bacteria and viruses in the gut. IGF-1, important for neonatal growth, is present at 4 to 62 times greater quantity in colostrum versus milk (Odle et. al., 1996). Additionally, colostrum is rich in casein, α-lactoglobulin, and ß-lactalbumin providing amino acids for protein synthesis and lean muscle development (reviewed in, Tizard, 2018).

Arguably, however, immunoglobulin is the most important component of maternal colostrum and the source of the majority of passive protection of the newborn. For the immunologically naïve bovine neonate, birth into an unsterile environment ensures immediate

challenge by environmental pathogens at the intestinal and respiratory mucosa. To ensure that maternal colostrum contains high levels of antibodies to these enteric and respiratory pathogens, many herds vaccinate the heifers and cows in the weeks prior to parturition with vaccines to benefit the calf. During this period, pregnant animals receive systemic vaccines to pathogens such as BRSV, PI3, rotavirus, coronavirus, and *E. coli*, the main respiratory and enteric pathogens of the neonatal calf. The immunoglobulin profile of colostrum synthesized during this period represents the systemic immunity of the dam and as such should provide broad-spectrum protection, representative of the antigen exposure obtained through vaccination of the mother as well as through natural exposure.

A number of physiological features help to ensure the immunoglobulins and other factors in the colostrum are protected from degradation in the digestive tract of the calf. These include reduced protease activity in the gut of the newborn calf and presence of trypsin inhibitors in the colostrum, both of which prevent digestion of proteins such as immunoglobulin (reviewed in Godden et al., 2019). Additionally, suckling stimulates the formation of the esophageal groove, a series of muscular folds from the reticulorumen that allow colostrum or milk to bypass the rumen, reticulum, and omasum, delivering milk directly to the abomasum of the neonate. Thus, colostral proteins quickly reach the small intestine intact where they are taken up by intestinal epithelial cells through pinocytosis. Immunoglobulins are transported across the lamina propria via the FcRn receptor which binds to IgG1, and released into the lymphatic circulation by exocytosis, ensuring the newborn receives a large infusion of maternal immunoglobulins (Barrington, Besser, Davis, et al., 1997; Staley et al., 1972).

Passive transfer of maternal immunity occurs during the first 24 hours after birth when the gastrointestinal (GI) tract of the neonate is “open” and is able to non-selectively absorb large molecular weight proteins. Intestinal permeability begins to decline rapidly after birth as the FcRn-bearing enterocytes are replaced by cells that do not express this receptor (reviewed in, Tizard, 2018). The process through which the intestinal cells gradually cease absorbing macromolecules is called “closure”. This process begins immediately after birth. The efficiency of Ig absorption across the intestinal epithelium decreases linearly until complete closure at 12-24 hours post-partum (Staley & Bush, 1985).

Good quality maternal colostrum (MC), capable of successful passive transfer contains 50 to >100g/L of immunoglobulin, equivalent to a Brix refractometer score of at least 23%. The IgG class comprises 85 to 90% of those antibodies, most of which is IgG1 (Barrington, Besser, Davis, et al., 1997; reviewed in, Foley & Otterby, 1978). Where IgG1 and 2 are equally represented in the serum, selective transport across the mucosal surface and into the udder results in 7x higher concentrations of IgG1 in the colostrum than in the serum (Larson et al., 1980). Re-secretion of the IgG1 from colostrum onto mucosal surfaces in the first weeks of life protects newborn calves from respiratory and enteric diseases. About 68% of the colostral IgG1 absorbed was shown to be re-secreted into the gut (Besser et al. 1988) and recent studies have demonstrated the appearance of IgG1 BRSV specific antibodies in nasal secretions collected from newborn calves fed colostrum containing BRSV specific antibodies (Ellis et al., 2018). Good quality maternal colostrum is an essential part of nutrition and disease management programs in cattle operations, providing broad-ranging nutritional benefits and immunological protection at multiple sites.

1.1.3 Milk and Passive Protection of the Suckling Neonate

At parturition, increasing prolactin levels and other endocrine changes signal a transition of the mammary gland from the state of serum IgG1 accumulation that defines colostrum production, to one of milk production (Barrington et al., 2001; Rincheval-Arnold et al., 2002). The hormonal changes that occur at parturition also signal plasma cells from the GI tract to migrate to the mammary gland via the blood, where their uptake is regulated by locally produced chemokines (Wilson & Butcher, 2004). The colostrum is consumed by the calf and/or removed by milking and/or the components present diluted by milk secretion. The proportions of the components of colostrum and milk are similar, but the scale changes markedly. However, while the sharp decrease in concentration of colostral components present in the milk may imply a simple dilution effect, milk is also a complex and unique biologic, nutritionally complete and potentially providing calves with a broad range of critical protections to pathogens local to the udder and, potentially, to the gastrointestinal tract (GI tract). However, due to physical and physiological changes in the calf, that protection differs from that offered by the colostrum.

While the predominant Ig in both colostrum and milk is IgG1, the concentration and proportion of Igs relative to other proteins changes dramatically in the transition from colostrum to milk production. At first milking, bovine colostrum contains approximately 14-16% (w/w) total

proteins compared to only 3% (w/w) in milk (reviewed in, Godden et al., 2019). Immunoglobulins comprise 80-90% of the total protein content of colostrum at 42-90 g/L, while in milk, that value drops to between 0.2 and 0.9g/L (reviewed in, Godden et al., 2019; Guidry, Paape, et al., 1980). In the transition of lacteal secretions, the most notable change is the increased representation of IgA in the milk, which increases from approximately 5% of colostral Igs to an average of 15% during lactation (Guidry, Paape, et al., 1980). Interestingly, only 30% of IgG and 10% of IgA in the milk is serum derived. The rest is derived locally from plasma cells in the udder (reviewed in, Hurley, 2003).

Due to the process of gut closure to non-selective absorption occurring so quickly and completely in the neonate, the antibodies in milk are not absorbed systemically. Instead, once they enter the gastrointestinal tract, some proportion provide direct protective benefits, agglutinating free antigen, preventing attachment until the invader can be digested or excreted (Arthington et al., 2002; Berge et al., 2009; Besser, McGuire, et al., 1988; Castrucci et al., 1984; Parreño et al., 2010; Saif et al., 1983). In numerous studies in piglets passive protection in the gut has been shown to be mediated by free IgA or IgG in the milk when high levels are maintained throughout lactation (Saif & Smith, 1985). That protection can be mediated by either Ig class and is facilitated by other co-factors found in the milk such as lactoferrin, lysozyme, fatty acids, and complement (Saif & Smith, 1985).

Milk potentially provides a different range of immunoglobulin protection to that of colostrum, due to the translocation of plasma cells from the gut into the udder at lactation, and immune responses following the potential exposure to non-systemic pathogens through the streak canal. Thus, antibodies in the milk may be directed primarily to pathogens found locally in the udder, and/or other agents targeting other mucosal surfaces. Mastitis, or the inflammation of the mammary gland, is caused primarily by pathogenic bacteria that move into the udder through the streak canal during the lactation period. Infiltration of mastitis-causing bacteria such as *Streptococcus*, *Staphylococcus* and coliforms result in acute upregulation of the innate immune system and an increase in immune components in the milk (Oviedo-Boyso et al., 2007). Protection from these pathogens require the presence of secreted antibody (Tzipori, 1981; Snodgrass & Wells, 1978). Variability in the immune components of milk can be attributed, in part, to the origin of Ig producing plasma cells and also the local antigen challenges faced by the mammary gland.

1.1.4 Antibody Classes, Subclasses and Specificities in Colostrum vs. Milk

While the nutritional differences between colostrum and milk are well established, questions around the disease sparing impact of the two lacteal secretions remain. In the face of pathogenic challenge, the specificity, amount, viability and types of antibody present at the challenge interface is key. This makes both secretions potentially interesting targets for further research as to how they are best utilized for improving calf health and performance.

IgG is the primary immunoglobulin in the serum of all mammals and it has a major role in antibody-mediated defenses. Antibodies are responsible for identifying foreign pathogens, which are then recognized by macrophages and neutrophils, enabling their eventual killing by phagocytosis or through the activation of complement. In cattle, this class can be further subdivided into the subclasses IgG1, IgG2, IgG3, and IgG4. Each subclass is comprised of slightly different amino acid sequences, affecting their electrophoretic mobility and contributing to different biological activities. For example, IgG2 agglutinates antigen particles and binds to a unique Fc receptor on macrophages and neutrophils, while IgG1 does not (reviewed in, Tizard, 2018). Normal adult bovine sera contains roughly an equal amount of IgG1 and 2, however, both bovine colostrum and milk are comprised primarily of IgG1 (reviewed in, Butler et al., 2015).

In mammalian species where IgG is transferred to the fetus prior to birth (ie., human, mice), IgA is the primary lacteal Ig component. In species where there is no transfer prior to parturition (ie. ruminants), IgG is the primary lacteal Ig. At parturition bovine colostrum is composed of approximately 24-80g/L IgG (90% of which is IgG1), 3-13g/L IgM, and 1-7g/L IgA (reviewed in, Tizard, 2018). Colostral immunoglobulins are concentrated in the mammary gland in late gestation by transportation across the mammary epithelium into the cistern of the udder (reviewed in, Hurley, 2003), at a rate of up to 500g IgG per week (Brandon et al., 1971). This translocation occurs via receptor-mediated processes; the neonatal Fc receptor (FcRn) for IgG1 and 2 (Guidry, Butler, et al., 1980), and the pIGR receptor for IgA and IgM (Mostov & Kaetzel, 1999). In colostrum, 100% of IgG and IgM and 50% of IgA are derived from the serum of the dam (reviewed in, Butler et al., 2015). Thus, the bovine neonate receives passive immunity through the colostrum that is mainly representative of the serum-borne antibody repertoire of the mother, heavily weighted to the IgG1 subclass.

In cattle, the primary Ig of milk remains IgG1. Unlike humans, IgA never becomes the

principle Ig although the proportion of IgA in the milk does increase substantially from that of the colostrum (reviewed in, Butler et al., 2015; Larson et al., 1980; reviewed in, Tizard, 2018). In contrast to antibody-rich colostrum, bovine milk is composed of only 0.5-7.5g/L IgG, 0.1-0.2g/L IgM, and 0.1-0.5g/L IgA. Although the total concentration of Ig is low in the milk, it is important to consider that the large volume of milk produced results in overall larger mass of Ig in milk relative to that in colostrum which is produced at much lower volume and for very short duration (Butler, 1981; Mach & Pahud, 1971).

There is currently debate regarding the origin of plasma cells in the mammary gland of cattle at lactation. Near parturition and during early and late lactation, hormonal changes signal the migration of gut-associated lymphoid cells (GALT) from the intestinal mucosa into the mammary gland (Gibson et al., 1991; Roux et al., 1977). The GALT is the largest organ of the immune system. It is comprised of Peyer's patches, lymphoid and myeloid cells in the lamina propria, as well as intraepithelial lymphocytes (Ishikawa et al., 2005; Spenser et al., 2007). The mechanism of trafficking GALT cells to the mammary gland is currently poorly understood but is believed to be provoked through changing serum concentrations of estrogen, prolactin, and progesterone (Barrington, Besser, Gay, et al., 1997; Barrington et al., 2001; Lascelles & McDowell, 1974; Rincheval-Arnold et al., 2002; Smith et al., 1971). The precursors of IgA-producing plasma cells have been identified as originating in the GALT and moving into the mammary gland during early and late parturition (Tanneau et al., 1999). However, these lymphocytes are phenotypically more similar to peripheral, rather than intestinal lymphocytes, leading some to question their origin (Bosworth et al., 1993).

The FcRn expression that facilitates IgG1 transfer coincides with the onset of colostrogenesis but decreases rapidly at the onset of lactogenesis. As FcRn receptor function declines and non-specific intestinal permeability abates, the steady, substantially decreased, presence of antibodies in the udder throughout lactation is believed to be mainly derived from the secretions of local plasma cells (Barrington, Besser, Gay, et al., 1997). Additionally, serum transport of IgA into the mammary gland in cows either does not occur or occurs at a very slow rate suggesting that IgA in cow milk is synthesized locally in the udder (Butler et. al., 1972, 1986). Additionally, organ culture studies have shown synthesis of both IgG1 and IgA in the mammary gland (Butler et. al., 1972; Sordillo & Nickerson, 1988). Local synthesis of Igs in the mammary

gland is also supported by immunohistochemical staining showing the location of IgA and IgG1 plasma cells in the mammary glands of ewes, sows and cows (Chabaudie et al., 1993; Collins et al., 1986; Fragkou et al., 2007; Mayer et al., 2002, 2005; Salmon, 1987, 1999; Salmon & Delouis, 1982). In sows, studies of radiolabelled Igs show 70% of IgG and more than 90% of IgA and IgM found in milk are synthesized locally in the mammary gland (Bourne & Curtis, 1973). In cows, some local synthesis of IgG1 has been demonstrated during lactation (Sheldrake & Husband, 1985). Additionally, the bovine udder has been shown to respond to stimulation through local immunization with the secretion of antibodies (Lascelles & McDowell, 1974; Smith et al., 1999). Although IgA and IgM are the primary antibodies produced. Systemic priming, followed by mammary gland immunization has also been demonstrated to lead to increased and sustained local antibody production capable of lasting through lactation (Watson & Lascelles, 1975). Ultimately it is believed that in milk, only 30% of IgG and 10% of IgA are serum-derived, the rest are thought to be locally-derived from resident plasma cells (reviewed in, Butler et al., 2015; reviewed in, Hurley, 2003).

The movement of lymphocytes between the GALT system and the mammary gland could provide a direct link between the broader maternal mucosal system and the secretory immune repertoire of the udder, potentially providing protective antibodies for any pathogens inhabiting the intestine or other mucosal tissues (Brandtzaeg, 2003, 2010; Hanson & Korotkova, 2002). This process may explain the differences in concentration, proportion and perhaps specificity of immunoglobulin between colostrum and milk as lactogenesis begins and the colostrum is replaced completely with milk.

Milk contains many of the components necessary to provide passive transfer of immunity to neonates if used as a colostrum replacement. However, due to the source of the antibody (primarily localized antibody production), milk may provide a different range of protection directed primarily to pathogens found locally in the udder and/or other mucosal surfaces (for example, *Streptococcus*, *Staphylococcus* and coliforms that enter through the streak canal, as well as to rotavirus and other mucosally-targeted pathogens) rather than the broad systemic protection afforded by colostral antibodies.

1.2. Failure of Passive Transfer of Immunity

Failure of passive transfer of immunity (FPTI) occurs when the concentration of IgG in the serum of the calf, considered consistent with neonatal health, is not reached prior to gut closure. This results in a physiological state which leaves the neonate susceptible to infection and disease (Heinrichs et al., 1994; *USDA APHIS | NAHMS Dairy Studies*, 2010, 2018). Passively acquired circulating antibodies provide protection against systemic infections, and through re-secretion onto mucosal surfaces, against enteric infections (Besser, Gay, et. al., 1988; Besser, McGuire, et. al., 1988; Saif & Smith, 1985, Ellis et al 2018). FPTI outcomes can be devastating to both individual producers and industry. FPTI can result in economic insufficiencies in commercial production, such as reduced first and second lactation milk production in heifers and even death losses (*USDA APHIS | NAHMS Dairy Studies*, 1996, 2018; Wells et al., 1996).

In the United States, pre-weaned dairy calves have an average mortality rate of between 5-11% (Urie et al., 2018; *USDA APHIS | NAHMS Dairy Studies*, 1996, 2018). Of these, up to 35% of calf mortalities are attributed to failure of passive transfer (FPTI) of maternal antibodies from colostrum (Brignole & Stott, 1980; Stott et al., 1979a; Urie et al., 2018; Wells et al., 1996). In a study by McGuire *et. al.*, 85% of calves less than three weeks old that died of infectious disease had failure of passive transfer of immunity (McGuire et al., 1976). There is a well-established relationship between BRD in calfhood and decreased milk production, reproduction and early culling (Stanton et al., 2012).

In dairy calves, passive transfer of maternal antibodies was traditionally considered successful and consistent with protection from mortality when neonatal serum IgG levels are greater than 10 mg/ml at 24 to 48 hr following birth ((Beam et al., 2009; Besser & Gay, 1994; Weaver et al., 2018). However recently the targets for passive transfer have been revised to the considerably higher levels considered necessary to protect from disease morbidity. New research recommends neonatal serum IgG level minimums of 15g/L, and higher serum IgG should be targeted for enhanced protection (Lombard et al., 2020; Urie et al., 2018). For beef calves the currently recommended target is even higher at 24-25g/L IgG (Dewell et al., 2006; Waldner & Rosengren, 2009). Achieving effective passive transfer of maternal immunity in neonates can be complicated as several factors impact the absorption of maternal Ig by the neonate. The mass of Ig consumed is critical; the mass is a function of the Ig concentration in the colostrum and the

volume consumed. The Ig concentration of IgG in maternal colostrum can vary greatly among and within farms, ranging anywhere from 7 to >150 g/L (Swan et al., 2007; *USDA APHIS | NAHMS Dairy Studies*, 2010). Good quality maternal colostrum, capable of providing adequate passive transfer, should be ≥50 g/L IgG (Godden, Haines, Konkol, et. al., 2009). In some studies up to 23-35% of colostrum does not meet that requirement (Quigley et al., 2013; Shivley et al., 2018; *USDA APHIS | NAHMS Dairy Studies*, 2010, 2018). In addition to low Ig concentration, low colostrum volume is not uncommon, especially among first calf heifers (Gavin et al., 2018). Providing calves with 3-4L of high-quality colostrum (at least 150g/L total IgG) within 1-2 hours after birth is the gold-standard for colostrum management programs. To this end, farms have traditionally stored and/or pooled excess maternal colostrum to supplement calves when maternal colostrum is deemed inadequate in quality or quantity to attempt to ensure all calves receive an adequate supply. However, this is a cumbersome process, prone to shortages and risks of pathogen contamination and inevitably results in highly variable colostrum management. Farms that pool colostrum from a variety of lactating dams are 2.2 times more likely to have calves with FPTI than those that do not pool colostrum (Beam et al., 2009). Another strategy to prevent FPTI is to supplement or replace maternal colostrum with commercial colostrum products (Haines et al., 1990), which potentially provides convenient, broad-spectrum immunity to calves.

1.3. Colostrum Replacement Products

The implementation of passive immunization strategies to prevent and treat infectious diseases in production animals is largely dependent on cost and effectiveness compared with existing solutions such as vaccination and treatment with antibiotics. Ideally, products must be easy to obtain and use, be broadly applicable, and integrate with existing vaccine and diagnostic management strategies. For antibody products, sourcing high volume, low cost, pertinent pathogen specific antibodies is of primary importance in the development of commercial products for managing passive immunization strategies in production facilities.

In 1950, Parrish et. al. (1950) first reported the composition of bovine colostrum (Parrish et al., 1950). Maternal colostrum was increasingly recognized as a critical source of nutrition for the neonate in addition to providing passive immunity by the absorption of Ig. As understanding of the biology of neonatal calf diets improved, it became apparent that protein concentration and, in particular, immunoglobulin concentration in the first colostrum feeding is more important than

the volume of colostrum fed and there was much important work conducted defining parameters of colostrum feeding, timing, and quality (Bush & Staley, 1980; reviewed in, Butler et al., 2015; Stott et al., 1979b, 1979c, 1979d). These studies laid the foundation for the many more recent studies that have culminated in the current recommendations (Beam et al., 2009; reviewed in, Godden & James, 2020; Lombard et al., 2020; Urie et al., 2018).

The development and availability of colostrum replacer (CR) products offers a standardized and convenient means of providing high quality and consistent passive protection and nutrition to calves while reducing the risk of pathogen exposure associated with unpasteurized lacteal fluids and thereby potentially breaking the transmission cycles of some infectious diseases. Supplementation with reputable commercial products provides the benefit of administering a known mass of IgG. The bovine immunoglobulin in these products can be obtained from a variety of sources including pooled colostrum, bovine serum, and/or milk whey, and could offer a broad range of protective antibodies to common bovine pathogens (Davis & Drackley, 1998; reviewed in, Godden & James, 2020; McGuirk & Collins, 2004).

Commercial colostrum products fall into two main categories, colostrum supplements (CS) and colostrum replacers (CR) (reviewed in, Godden & James, 2020). Supplements are designed to supplement maternal colostrum. These products do not have to show prevention of FPTI. As such, each portion of CS product provides between 50 to 100g in 1-2 L volumes (reviewed in, Godden & James, 2020). CR products must contain sufficient IgG mass to ensure adequate passive transfer without access to maternal colostrum, typically 150-200 g IgG.

Studies have shown that when administered within the first hours after birth and in high enough Ig mass (150 to 200 g IgG), commercially prepared CR products, similarly to pooled maternal colostrum, can result in acceptable passive transfer levels and absorptive efficiencies (Arthington et al., 2000; reviewed in, Cabral et al., 2012; Foster et al., 2006; Godden, Haines, & Hagman, 2009; Godden, Haines, Konkol, et al., 2009; Pithua et al., 2009; Place et al., 2010; Priestley et al., 2013; Quigley & Bernard, 1996; Santoro et al., 2004; Shea et al., 2009). High quality colostrum collected and pooled from North American dairy farms have antibodies to all common, endemic pathogens such as *E.coli*, rotavirus, and BRSV through natural exposure and/or through vaccination programs. In some cases, commercial products may prove superior to maternal colostrum (MC). In one study, calves fed a CR product had a reduced risk of testing

positive to infection with *Mycobacterium avium* subsp. *paratuberculosis* (7.6% test positive, odds ratio 0.52 [0.27 to 1.003]), compared to maternal colostrum-fed control calves (11.9% test positive), suggesting that MC may be a vector for *Mycobacterium avium* subsp. *paratuberculosis* (Pithua et al., 2010).

Colostrum replacer (CR) products should enable the feeding of a known IgG mass to ensure the magnitude of passive transfer of immunity in calves. However, the results of colostrum replacer trials have been mixed with many early to market products failing to provide the minimal 10g/L calf serum IgG (Quigley et al., 2001; Swan et al., 2007). In a trial of 12 dairy herds, 239 calves were fed a serum -derived CR product containing 125g of IgG vs 218 calves fed maternal colostrum. Both groups were then supplemented at 8 to 12 hours with 1.9 L of MC, for the MC-fed group, and 1.9L of milk replacer supplemented with 45g plasma-derived CS for the CR-fed group. CR-fed calves had significantly lower serum IgG concentrations at 5.8g/L compared to MC fed calves at 14.8g/L. In this study, preweaning morbidity and mortality rates were the same and high for both groups; 59.6% morbidity and 12.4% mortality for CR fed and 51.9% morbidity and 10% mortality for MC fed calves (Swan et al., 2007). In contrast, Pithua et. al. found that calves fed a colostrum-derived CR containing 200g IgG were significantly less likely to have failure of passive transfer of immunity than calves fed 3.8L of 21g/L pooled maternal colostrum. In this trial, only 11% of calves fed CR experienced FPTI, while 70% of MC fed calves had FPTI (Pithua et al., 2013). The magnitude of this discrepancy however is due to the atypically low IgG of the pooled maternal colostrum fed to the control group. Further, in a national survey where the average maternal colostrum contained 64.3g/L maternal colostrum, calves fed still experienced 23% FPTI (Smith, 2014), which was significantly greater than the neonates fed colostrum derived CR product in the above referenced study.

In general, studies of commercial products reporting the most success in achieving $\geq$10g/L passive transfer have fed $\geq$100g IgG in the first hours following parturition (Mee et al., 1996; Quigley et al., 2001). In one such study, calves were fed 100g or 200g IgG from CR products, vs 3.78L MC, calf serum IgG levels at 24 hours were reported as 11.6, 16.9, and 27.2 g/L total IgG, respectively (Foster et al., 2006). More recent studies emphasize the benefits of feeding larger IgG mass to achieve higher serum IgG concentrations (reviewed in, Godden & James, 2020; Hare et al., 2020; Lombard et al., 2020; Urie et al., 2018). In fact, data from the USDA's N.A.H.M.S.

Dairy study (2018), report greater probability for survival in calves with passive transfer levels >15g/L IgG (reported in: Urie et al., 2018). Other studies report decreased susceptibility to respiratory infections at >15g/L total IgG (Furman-Fratczak et al., 2011), and reduced mortality rates in calves at passive transfer levels as high as 20g/L total IgG (Chigerwe et al., 2015).

While it is well accepted that passive transfer outcomes are highly dependent upon IgG mass fed, another potential explanation for differential success in CR replacement studies is the source of bovine immunoglobulin. Colostrum replacer ingredients are divided into 4 categories: dried colostrum products, milk/whey protein products, plasma or serum-based products, and products that are a combination of some or all of the previous categories. Bovine serum is Ig-rich, affordable and readily available from slaughterhouses. Adult bovine serum is composed of an equal ratio of IgG1 and IgG2, in comparison to the IgG1-rich colostrum. Immunoglobulin comprises approximately 20% of spray-dried, blood plasma dry matter (Pierce et al., 2005; Quigley & Drew, 2000), that can be further purified and concentrated (Lihme et al., 2010). However, there is evidence that products formulated from serum have lower apparent percent efficiency of absorption (AEA) of immunoglobulin than those made from natural colostrum (Arthington et al., 2000; Brakefield et al., 2010; reviewed in, Cabral et al., 2012; Godden et al., 2016; reviewed in, Godden & James, 2020; Godden, Haines, & Hagman, 2009b; Godden, Haines, Konkol, et al., 2009; Morrill et al., 2010; Pithua et al., 2013; Santoro et al., 2004; Shea et al., 2009). Apparent efficiency of absorption of immunoglobulins (AEA%) is a metric of evaluating passive transfer efficiency, determined using the amount of IgG fed, calf serum IgG level, and calf body weight (Quigley et al., 2002). In the fifth and sixth editions of the textbook Large Animal Internal Medicine (2014, 2020), Godden published a review of study results from products tested up to 2018 and these data support the suggestion that products produced from colostrum have superior absorptive characteristics relative to those that utilize immunoglobulin from other sources (reviewed in, Godden & James, 2020; Smith, 2014). It is therefore conceivable that the source of Igs and the increased proportion of IgG1 found in colostrum-derived replacer products provide immunological advantages over serum-derived products, potentially resulting in improved calf health and other long-term benefits. However, studies testing this hypothesis have not been conducted.

Given the purported superior efficacy of colostrum-based CR products, the collection of excess dairy colostrum is a logical source of bovine-specific antibodies. However excess dairy colostrum is limited in supply, expensive to collect and process. Consequently, other sources of bovine immunoglobulin have been sought to create colostrum "formulas". These include the use of milk whey, a by-product of cheese manufacture (Haines et al., 1990). The ratios of Igs and Ig subclasses in milk are similar to those of colostrum and while the levels of IgG are low in milk compared to colostrum (0.5-1.0g/L vs 20-200g/L, respectively) there are methods to concentrate the IgG thereby providing a relatively inexpensive source of immunoglobulin (Hurley & Theil, 2011; Siso, 1996; *USDA APHIS | NAHMS Dairy Studies*, 2010, 2018). Thus, some commercial products for colostrum replacement may contain immunoglobulin derived from milk rather than colostrum. Currently there are no studies that have examined the specificities of antibodies in milk whey derived products to investigate whether these differ from those in colostrum and/or that this practice may result in products with different efficacy in newborn calves. One study of the whey-based product Colostrx (Colostrx CR Colostrum Replacer; Agrilabs, St. Joseph, MO) claims to provide a comparable level of clinical protection to colostrum in an *E. coli* F5 (K99) challenge model (Harman et al., 1991). *E. coli* is the most common cause of septicemia and diarrhea in calves, causing substantial financial losses for individual farmers as well as commercial beef and dairy industries (Kolenda et al., 2015). Given the belief that milk antibody production is localized in the udder by GALT cells that migrate from the intestine, coincident with the onset of lactation, milk antibodies could potentially be used to provide protection to common and economically important pathogens such as *E. coli*, in the form of increased antibody levels to enteric and localized udder pathogens.

Among the concerns arising with the use of CR products has been the capacity of colostrum and colostrum replacement products to transmit disease. Standard milk pasteurization conditions may result in viscous, coagulated colostrum due to its higher protein concentration. However, calves fed colostrum heat-treated for 1 hr at 60°C have shown successful outcomes in the form of successful passive transfer and/or decreased morbidity, particularly due to diarrheal agents. In one study of 1093 calves, half were fed fresh colostrum and half were fed heat-treated colostrum. The calves fed heat-treated colostrum had high passive transfer levels and better AEA than those fed fresh colostrum (18mg/ml total IgG compared to 15.0mg/ml, respectively). Additionally, in this study calves fed pasteurized colostrum were less likely to require treatment events, particularly

due to scours, than those fed fresh colostrum (30.9% vs 36.5%, respectively) (Godden et. al., 2012).

Colostrum products are almost always manufactured to a dry powder that is reconstituted with water prior to feeding. Chelack *et. al.* (1993), compared three methods of drying colostrum, concluding that spray-drying was the most cost-effective method. Calves fed a total of 126g IgG in two 1L feedings of either maternal colostrum or spray-dried colostrum had equivalent serum IgG concentrations at 48h (10.57g/L and 11.6g/L, respectively). There are few subsequent published studies that have addressed the best methods for producing commercial products as the information is regarded by manufacturers as proprietary. Regulatory agencies currently allow licensed colostrum products to be either heat treated or irradiated as long as the safety of the methodology is demonstrated to the agencies. However, these results are proprietary.

Colostrum products that are licensed by the USDA and CFIA with claims to prevent FPTI are considered in the class of therapeutics termed "veterinary biologics" which also comprises vaccines and antibody products such as anti-sera. Only CR products derived from colostrum are eligible for inclusion and allowed to have these claims. All others are marketed as "feed" products although many manufacturers typically market these products as *de facto* colostrum replacers with claims to prevent FPTI. Licensed CR products must be produced in federally inspected plants and must undergo regular purity and potency testing to ensure they do not exceed specified bacterial counts and are free of coliforms, *Salmonella* spp., and fungi. They are also tested for efficacy so that within 24hrs post-treatment, 90% of animals that receive this product must have total serum IgG measurements at least equal to the USDA-APHIS approved IgG Species Standard (*Canadian Food Inspection Agency*, n.d.; *USDA* 2007, 2014). Colostrum for these products is collected from certified dairies, frozen, then later thawed, pooled, heat treated or treated with ionizing radiation, spray dried and packaged. Aside from the loss of cellular components such as bacteria and leukocytes, these products will be very similar in composition to fresh maternal colostrum.

Colostrum replacement products derived from milk whey or blood cannot be licensed by federal regulatory agencies (CFIA and/or USDA) for use in newborn calves to prevent FPTI and are not legal in Canada however they may be sold as animal feed without a claim for providing immunoglobulin or passive immunity in the USA. In the US there are few FPTI licensed products; the vast majority of products promoted as "colostrum replacers" are actually animal feed products

and do not fall under USDA regulations for antibody products. While it is illegal to make passive transfer claims for such feed products this is not enforced. Licensed products are strictly regulated and may only contain colostrum as an antibody source, while "feed" products may contain antibodies sourced from colostrum, milk and/or blood.

Milk sourced antibodies, in contrast to blood, have many of the characteristics of colostral immunoglobulin. The first colostrum product widely marketed commercially (Colostrx) contained the immunoglobulin found in the milk whey fraction of cheese manufacture (Haines et al., 1990). This trade-name product is a feed rather than a FPTI licensed product and as such has undergone several subsequent iterations in ingredient source and the source of the immunoglobulin in this and other feed products is difficult to determine, and subject to change as manufacturers find alternative sources of ingredients. It is currently unknown how many of the many unlicensed CR products sold in the USA have milk whey as an immunoglobulin source.

While it is relatively simple to measure the total mass of IgG administered in CR products and the resulting levels of passive transfer compared to feeding maternal colostrum there have been few studies focused on understanding the specificities of the antibody delivered to calves fed CR products. Chamorro et al (2014), reported that when calves were fed a colostrum based replacer (CR) with 208.5g IgG, vs those fed maternal colostrum with 277.4g IgG, the CR product provided calves with more uniform levels and predictable duration of persistence of antibody to common bovine respiratory viruses, while those fed MC had greater variation and overall shorter duration of detection of antibodies (Chamorro et al., 2014). Thus, feeding commercial products derived from high quality pooled colostrum may improve and provide a longer and more uniform level of protection.

Colostrum replacer products are an increasingly accessible option on farms with limited labour and/or access to colostrum reserves, those with high numbers of heifers producing low volume and/or quality colostrum, or producers trying to break the cycle of transmission of many common bovine diseases. As the utilization of colostrum replacer products increases, research into the efficacy of these products has demonstrated that mass of IgG alone is not a sufficient predictor of either optimal passive transfer or calf performance. There have been no studies that have attempted to examine the differences in the source and/or the specificity antibodies from differing sources to understand if this may impact the utility of these products.

2. TRANSITION STATEMENT

Early life immune status is an important variable contributing to health and future performance of calves. Colostrum is the vehicle through which important immune factors including immunoglobulin, growth factors and nutrients are transferred from the dam to the neonate. Following birth, the calf's ability to absorb macromolecules is quickly reduced, and by 24 hours the gut is almost completely closed to further uptake. Thus, the first feeding is the single most important meal a calf will consume in its lifetime. In a dairy calf, the initial feeding should provide at least 150-200g of Immunoglobulin G (IgG). When the dam does not provide sufficient quality or quantity of colostrum to the neonate, or for control of disease transmitted in colostrum, commercially available colostrum replacers (CR) products may be an appropriate alternative. Colostrum replacer products can be made from various sources, including colostrum, serum and/or milk whey. In colostrum, antibodies and co-factors are primarily serum-derived and the immunity should be representative of the history of systemic antigen exposure of the dam. In contrast, immunoglobulins in milk should be primarily derived from localized plasma cells, which migrate from the intestinal mucosa at parturition and during early and late lactation. Milk could therefore provide a different range of protection and may be primarily to pathogens found locally in the udder and mucosal surfaces (for example, *Streptococcus* and coliforms that enter through the streak canal). Calves fed milk sourced immunoglobulin, or CR products enhanced with milk whey IgG to increase total IgG content, may have lower specific antibody levels to systemic pathogens, compared to those fed colostrum. Because there is interest in the complete or partial supplementation of colostrum replacer products with whey derived from milk, it is important to characterize milk whey antibodies to determine if they are likely to be of equal or sufficient benefit compared to colostral antibodies.

3. OBJECTIVES

The use of commercial colostrum replacer (CR) products enables producers to break the cycle of many common calfhood diseases transmitted in the colostrum, such as Johne's disease. Use of commercial products also gives producers control over the quality and quantity of colostrum fed at parturition, facilitating the successful passive transfer of immunity in newborn calves. Identifying and understanding the specific antibodies of potential sources of bovine-specific immunoglobulins could influence their use and potentially lead to better quality CR products and improved calf health and disease management outcomes. To further understanding in this area, the following objectives were addressed in this thesis:

- The overall objective was to determine if there is differences in the magnitude of the specific antibodies in IgG found in serum, milk and colostrum in a group of normal dairy cows.
- The specific objective was to estimate the magnitude of antibodies to a selection of pathogens including some considered to be found systemically and others considered to be more localized to the gut mucosa and udder
- An additional objective was to determine if there are apparent differences among the specific antibodies present in commercially available colostrum products

4. "COMPARISON OF THE LEVELS OF SELECTED SPECIFIC ANTIBODIES IN THE IgG OF COLOSTRUM vs. MILK AND SERUM IN DAIRY COWS (*BOS TAURUS*)"

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John A. Ellis, University of Saskatchewan, Veterinary Microbiology. Supervisor, study design, method development support.

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Abstract:

In cattle (*Bos taurus*), the immunologically naïve newborn calf receives immune factors, growth factors and nutrients from the dam via the colostrum. Colostral antibodies are primarily serum-derived and provide broad-spectrum protection representative of the systemic immunity of the mother. In contrast, immunoglobulins in milk are primarily derived from udder localized plasma cells, which migrate from the intestinal mucosa at parturition and during lactation. It is an increasingly common practise that newborn dairy calves are fed commercial colostrum replacement products containing IgG sourced from colostrum, milk and/or serum in addition to or instead of maternal colostrum. We hypothesized that IgG derived from milk will have lower levels of antibodies to systemic pathogens, compared to serum and colostrum. We sampled serum and colostrum (1-2hr post partum) and milk (day 5 post partum) from 24 dairy heifers or cows, measured total IgG using a radial immunodiffusion assay (RID) and determined specific antibodies

(IgG class) for a variety of systemic, gut and udder-associated pathogens (ie. bovine respiratory syncytial virus (BRSV), bovine herpes virus (BHV-1), parainfluenza 3 (PI3V), *Streptococcus uberis*, *Staphylococcus aureus*, *E. coli* F5 (K99), rotavirus and bovine coronavirus, via enzyme-linked immunosorbant assay (ELISA). We also performed additional ELISAs to investigate the IgG1 and IgG2 subclass antibodies for BRSV, rotavirus and *S. uberis*. Wilcoxon signed rank tests (with Bonferroni correction, P<.017) indicate that colostrum derived IgG contains more antigen specific antibodies to BRSV (IgG H+L), PI3V (IgG H+L) *S. aureus* (IgG H+L), *E. coli* F5 (K99) (IgG H+L), and rotavirus (IgG H+L and IgG1) and BCV (IgG H+L), than the IgG from milk. In comparison to the serum, colostrum is higher in BRSV (IgG H+L and IgG1), BHV-1 (IgG H+L), PI3V (IgG H+L), *E. coli* F5 (K99) (IgG H+L), *S. uberis* (IgG H+L) and rotavirus (IgG1). Milk IgG contains more specific antibody to BRSV (IgG H+L and IgG1), BHV1 (IgG H+L), PI3V (IgG H+L), and rotavirus (IgG1) when compared to serum. Colostrum derived IgG delivers more specific antibodies to most endemic pathogens compared to the IgG found in milk or serum. However, milk IgG, similarly to colostrum has higher amounts of specific IgG1 and delivers a similar spectrum of antibodies. Thus, bovine milk IgG may be a superior source for the newborn calf compared to serum sourced IgG.

4.1 Introduction:

Ruminants, including cattle, are born almost agammaglobulinemic; neonates receive immunoglobulin from the dam in the “first milk”, or the colostrum (Gullickson et al., 1942). Colostrum is created during the five-week dry (non milking) period leading up to calving when the upregulation of lactogenic hormones signals the accumulation of lacteal secretions and serum components in the udder. It is depleted as the udder is suckled or milked in the first 1 to 3 days postpartum (reviewed in, Butler et al., 2015).

Good quality maternal colostrum (MC), capable of successful passive transfer of immune protection to the neonate, contains 50 to >100g of immunoglobulin (reviewed in, Godden & James, 2020; Shivley et al., 2018; Waldner & Rosengren, 2009). The class of antibodies found in colostrum is 85 to 90% IgG (reviewed in, Tizard, 2018). The major IgG subclass in colostrum is IgG1 that is primarily serum-derived. Thus the antibodies in colostrum represent the recent maternal immune response to both environmental pathogen exposure and vaccination history,

providing broad range protection to most ubiquitous calfhood pathogens (reviewed in, Butler et al., 2015 Barrington, Besser, Davis, et al., 1997; reviewed in, Foley & Otterby, 1978). Ensuring good quality colostrum is an essential component of nutrition and disease management programs in cattle operations.

Colostrum replacement (CR) products have been commercially available for over three decades and can be used to supplement, or totally replace, maternal colostrum when maternal supplies are of limited quantity, poor quality, or are considered at risk of disease transmission (reviewed in, Godden et al., 2019). Studies of CR feeding trials have mixed results, but in general feeding 150-200g total IgG within the first few hours after birth results in successful passive transfer of immunity to neonates (reviewed in, Cabral et al., 2013; reviewed in, Godden & James, 2020). While the obvious source of immunoglobulin for CR products is colostrum, even dairy cows produce colostrum in only modest quantities, making it expensive and difficult to obtain.

A more readily available source of bovine IgG for CR products is plasma obtained during slaughter. Plasma derived IgG is, however, approximately equal amounts of IgG1 and IgG2 subclasses. IgG2 cannot be re-secreted at mucosal surfaces; a critical protective advantage of IgG1 which occurs at much greater abundance than IgG2 in colostrum (Besser, Mcguire, et al., 1988; Ellis et al., 2018). Thus, about half the IgG mass in blood sourced products may be of limited value in protection of mucosal surfaces of the neonate.

Immunoglobulins in milk are far less abundant than in colostrum and are primarily derived from udder localized plasma cells, which migrate from the intestinal mucosa at parturition and during early and late lactation (only about 30% of IgG in milk is serum-derived) (reviewed in, Butler et al., 2015). Thus, milk likely contains IgG with specific antibodies primarily to pathogens found locally in the udder and at other mucosal surfaces.

Milk derived IgG has been used as a source of immunoglobulin for CR products (Harman et al., 1991; Mee et al., 1996). However, the question of differences in the specificities of the antibodies compared to those found in the IgG of serum/plasma or colostrum has not been examined. The dairy industry harvests milk in large quantities and milk whey containing IgG is widely available as a by-product of cheese production. While the immunoglobulins in milk are at relatively low concentration, the volumes produced result in an overall large yield of antibodies that can be harvested and concentrated. In addition, in contrast to serum, the composition of IgG

subclasses in milk is identical to colostrum (primarily IgG1), allowing for re-secretion of milk-derived antibodies at mucosal surfaces (reviewed in, Butler et al., 2015; reviewed in, Hurley & Theil, 2011).

The goal of this study was to determine if there are differences in the amounts and specificities of antibodies in the IgG harvested from colostrum, serum and milk in a group of post parturient dairy cows to better understand if there can be benefits or disadvantages in using these IgG sources for passive immunity in newborn calves.

4.2 Materials & Methods:

Sample Collection:

This study was approved by the University of Saskatchewan Animal Care committee (AUP *#009CATA2018).* Samples were collected from all cows and heifers calving within a two-week period at a commercial dairy farm in New York State. The 19 Holstein and 5 Brown Swiss dairy cows were maintained according to the routine management procedures of the farm. Serum and colostrum samples were taken at first milking, within 1-2hr of calving. Blood samples were collected from the tail vein into 10ml vacuum tubes. Blood was centrifuged at 1400krpm x 10 min and serum harvested into new 10ml tubes and frozen at -20C. Concurrently, a 10-25ml sample of first milking colostrum was collected and frozen at -20C. A milk sample was similarly collected from each cow on day 5 following parturition and also stored frozen. All samples were shipped frozen, then thawed and aliquoted on arrival at the testing laboratory.

Measurement of IgG Concentration in Colostrum, Milk and Serum:

IgG concentration was determined for each biologic using a single radial immunodiffusion assay (sRID) (Chamorro et al., 2017; Chelack et al., 1993b). Briefly, serum (diluted 1:4), milk (undiluted) and colostrum (diluted to a minimum 1:4) were diluted in 1x PBS and 4 uL samples deposited in wells punched in an agar matrix infused with antiserum to bovine IgG (Bethyl Laboratories Inc., Montgomery, Texas, USA). After 18 hours incubation at room temperature, a calibrating viewer (Transidyne General Corporation, Ann Arbor, Michigan, USA) was used to measure the diameter of the immunoprecipitin rings formed around the samples, which was compared to the regression curve of a serially diluted serum standard containing a known amount

of bovine IgG (Midland Bioproducts, Boone, Iowa, USA). Total serum proteins and Brix values were measured using a refractometer (Palm Abbe - Misco, Solon, Ohio, USA).

Specific Antibody Values in the IgG of Colostrum, Milk and Serum:

Enzyme-linked immmunosorbant assays (ELISAs) were performed as previously described (Durham & Sillars, 1986; Ellis et al., 2013) with modifications, to estimate the magnitude of specific IgG antibody (H+L) for BRSV, PI3V, BHV-1, rotavirus, bovine coronavirus, *Escherichia coli* F5 (K99), *Streptococcus uberis* and *Staphylococcus aureus* in the colostrum, milk and serum samples. Additional ELISAs for IgG1 and IgG2 subclasses were performed for BRSV, *S. uberis* and rotavirus. These targets were chosen to represent the respiratory, mastitis-associated and GI-associated pathogen groups.

Prior to testing, utilizing the results of the sRID assays for IgG concentration, each sample was adjusted to 0.5 g/L IgG, such that all samples were tested in each assay at the same IgG concentration. For testing, samples were further diluted so that the expected range of values fell within the linear portion of a standard curve derived from serial dilutions of a positive control serum. Briefly, 96-well plates (Immulon IV; Thermofisher, Waltham, Massachusetts, USA), were coated with a carbonate coating buffer containing a heterogenous antigen preparation, purified from pathogen-infected cell lysates, as well as control uninfected lysates prepared identically. Diluted samples were applied in 100uL volumes to the antigen-coated 96-well plate. Following incubation and washing, a horseradish-peroxidase conjugated recombinant protein G from *Streptococcus* sp. was applied for IgG (H+L) detection (Zymed; Thermofisher, Waltham, Massachusetts, USA), or specific antiserum for IgG1 or IgG2 detection (Bethyl Laboratories Inc., Montgomery, Texas, USA), and the plates incubated. The HRP conjugates were removed by washing and a single component 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate applied (Kirkgaard & Perry Laboratories, Gaithersburg, Maryland, USA). The enzymatic reaction was stopped with 1% SDS (Sigma Aldrich; St. Louis, Missouri, USA). The colour changes (which were proportionate to the levels of antibodies) were measured spectrophotometrically at 405nm and compared to an immune positive control serum and negative control fetal bovine serum concurrently tested on each plate. Units were reported as the percent of the optical density (OD) of the positive control serum minus that of the uninfected cell lysate (Durham & Sillars, 1986; Ellis et al., 2013; Engvall & Perlmann, 1972).

In order to estimate the absolute differences in the magnitude of ELISA unit values, a standard curve for each assay was created by serially diluting the positive control serum for each antigen (Appendix A). These curves were examined to approximate the change in units that would equate to one doubling dilution (twice the amount of antibodies) in the test. A steeper standard curve results in a smaller change in units for one doubling dilution, while the converse is true for a shallower standard curve where there is a greater change in units equivalent to a doubling dilution. For example, for the BRSV (IgG) assay, a change of 10 units is approximately equivalent to a doubling dilution (twice the amount of detected antibody), while the much shallower standard curve of a PI3V titration results in 30 units equating to a doubling dilution. Doubling dilution values for each target were then used to estimate differences between samples for each biologic (colostrum, serum and milk) using the following calculation (ELISA units biologic A – ELISA units biologic B)/target-specific doubling dilution value.

Statistical Analysis:

SPSS version 25 statistical software (SPSS v. 25, IBM, Armonk, New York, USA) was used for statistical analysis of ELISA unit values for each assay. A Shapiro-Wilks test revealed the ELISA data were not normally distributed, thus the data obtained for the antibodies in the IgG of the three biologics (colostrum, serum and milk) were compared using the non-parametric Friedman Analysis (ANOVA), with a significance level of $P<0.05$. Data for paired biologics were compared using the non-parametric Wilcoxon test, with Bonferroni correction and a significance level of $P<.017$.

4.3 Results:

IgG Concentrations in Colostrum, Milk and Serum:

The concentration of IgG (g/L) from biological samples collected from each animal was measured using RID assay as described in the material and methods section. Results from this assay showed that colostrum samples had the highest IgG concentration with a mean value of 94.6 g/L, and showed a wide concentration range from 12.4g/L to 201.7g/L (Table 1). The IgG concentration in the milk was much lower (mean value 1.2g/L), but the range was less pronounced, spanning from 0.5 to 2.8g/L. Serum samples had mean value of 24.3 g/L and a range from 10.9

g/L to 52.1g/L. The total protein, total IgG and Brix scores were consistent with expected ranges in post partum dairy animals (Shivley et al., 2018). Table 1 shows the IgG concentrations in the 3 fluids collected from each of the 24 animals. Though serum values were much higher than those of milk, the two biologics mimicked each other in proportion, i.e., if serum IgG was high then milk IgG values also tended to be high. This pattern was less consistent in the colostrum, where IgG was present in much higher levels than serum or milk. However, in general if IgG in the colostrum was very high, the same were true of milk and serum.

Specific Antibody Unit Values in IgG of Colostrum, Milk and Serum of Individual Cows and Heifers:

IgG values in colostrum and CR products are generally reported as g/L, therefore while all biologics were normalized to 0.5g/L for testing (to accommodate for low milk values), they were mathematically standardized to 1g/L for ease of reporting and discussion purposes.

Figure 1a and b shows the antigen specific ELISA unit values found in the colostrum, serum and milk IgG for the group of animals, for each assay. Appendix B shows the data for each individual animal. Antibodies to BRSV (IgG) and BHV1 (IgG) generally followed a similar pattern among animals across all three biological fluids (Figure 1a). For these agents, colostrum IgG was usually the highest and serum IgG the lowest in antibodies. In general, however, if an animal tested high for antibody to these pathogens in the serum IgG, that value was also high in the IgG of the colostrum and milk. Colostrum and milk were higher for the IgG1 subclass antibodies than the serum, with colostrum higher than milk (Figure 1b). In general, there was agreement in the amplitude patterns among the three biological fluids for IgG1 antibodies. Serum was always highest in IgG2 antibodies, followed by milk (Figure 1b). These two biologics tended to follow the same trend in representation of the IgG2 subclass antibodies, while colostrum did not follow the same pattern. In contrast, antibodies to PI3V, another viral pathogen associated with respiratory infection, were more variable (Figure 1a). In the majority of animals, antibody values were lowest in the serum IgG and highest in the colostrum IgG. Milk IgG antibodies for PI3V were more variable and did not follow a consistent pattern, Low values in the serum IgG were not always predictive of low values in the milk IgG.

Aside from one animal, specific antibodies to *S. aureus* (IgG) were much higher in the serum IgG than in the colostrum IgG, and even more so than in the milk IgG (Figure 1a). In general, however, when antibodies to this agent were high in the serum IgG they tended to be high in the colostrum IgG. In contrast, antibodies to *S. uberis* were variable in the IgG in each of the biologics, though colostrum IgG often had the highest level of this antibody (Figure 1a). When considering IgG1 and 2 subclass antibodies for this pathogen, IgG2 was primarily found in the serum IgG and levels of both subclasses fluctuated without an obvious pattern in the IgG of the lacteal fluids (Figure 1b).

E. coli F5 (K99) (IgG) antibodies were most often lowest in the milk IgG and highest in the colostrum IgG (Figure 1a). However, within individual animals there was no apparent relationship in the *E. coli* specific antibodies levels in the IgG among the three biologics.

Rotavirus specific antibodies were consistently highest in the serum IgG, followed by the colostrum and then milk IgG (Figure 1a). There were however high levels (relative to the positive control) of rotavirus antibodies in the IgG of all three of the biologics from this group of animals. Colostrum IgG rotavirus antibodies were primarily composed of IgG1 (Figure 1b). While serum rotavirus antibody appeared to be mostly IgG2 (Figure 1b). In individual animals when serum IgG rotavirus antibodies were low in IgG1, this subclass was correspondingly high in the colostrum IgG. Levels of rotavirus specific IgG1 and IgG2 antibodies were consistently lowest for the milk IgG, but when IgG2 was lower in the serum IgG, milk values were higher.

Bovine coronavirus (IgG) antibodies were most often highest in the colostrum IgG, but there was no apparent relationship among the levels in the serum, milk or colostrum IgG of individual cows (Figure 1a). However, for BCV (IgG) and rotavirus (IgG), antibodies in the IgG of samples from individual cows follow a similar pattern for amplitude (i.e., if antibodies were high in the IgG of one biological fluid from that animal, they tended to be high and vice versa), but it was highly variable which IgG would have the highest levels.

Statistical Analysis of Differences in Specific Antibodies in the IgG of Colostrum, Milk and Serum:

All data was nonparametric by Shapiro-Wilks testing, so statistical analysis via Friedman test was employed to determine significance of differences in the levels of specific antibodies in the IgG among the three biological samples from this group of 24 cows and heifers. In this test, the value of each sample for a given test is given a rank and the squared sum of the ranks compared to determine their statistical relationship. There were statistical differences in the antibodies found in the IgG from the three biologics for all targets examined except *E. coli* F5 (K99) and *S. uberis* (IgG1). Further testing by Wilcoxon signed-rank test with Bonferroni correction agreed that there were no significant differences among the paired biologics for *S. uberis* IgG1, however subsequent analysis indicated the significance of differences between *E. coli* F5 (K99) levels in the three biologics was likely obfuscated by the scale of some of these differences between pairs of samples (Figure 2a-c).

A Wilcoxon Signed Rank Test, with Bonferroni correction ($P<.017$) was chosen to compare the distributions of the population means of pairs of related biologics (Figure 2). In this figure the horizontal axis represents the Z-score of the test. Values to the left of 0 are negative, while those to the right are positive. The critical value, or Z-score of this test in a comparison of two biologics, with the corrected P-value of 0.017 is +/-2.385. Values greater than 2.385, or less than -2.385 denote statistically significant differences between the two biological samples being compared.

Figure 2a compares the mean ELISA antibody units for this group of animals in colostral IgG to those in milk IgG. Colostrum IgG is statistically higher in antibodies for most targets tested including BRSV (IgG), PI3V (IgG), *E. coli* F5 (K99) (IgG), *S. aureus* (IgG), rotavirus (IgG and IgG1), and bovine coronavirus (IgG). Additionally, antibodies for BHV1 (IgG), *S. uberis* (IgG and IgG1) and BRSV (IgG1), were higher in the colostrum IgG than milk IgG, but not significantly so. Milk IgG was higher in IgG2 antibodies to BRSV, *S. uberis* IgG1 and rotavirus but not statistically so.

Figure 2b compares mean ELISA antibody units in colostrum IgG to those found in serum IgG. Colostrum IgG was statistically higher in specific antibodies to BRSV (IgG and IgG1), BHV-1 (IgG), PI3V (IgG), *E. coli* F5 (K99) (IgG), *S. uberis* (IgG) and Rotavirus (IgG1) and while not statistically significant was also higher in antibodies to *S. uberis* (IgG1) and BCV (IgG). Serum

IgG was statistically higher in IgG2 antibodies to all three targets tested (BRSV, *S. uberis* and rotavirus). Serum was also significantly higher in IgG antibodies to *S. aureus* than the colostrum.

Figure 2c compares mean ELISA antibody units in serum and milk IgG. The results of this analysis were more mixed. In comparison to serum, milk IgG had statistically more antibodies to BRSV (IgG and IgG1), BHV-1 (IgG), PI3V (IgG), and rotavirus (IgG1). While not statistically significant, milk IgG is also higher in specific antibodies to *E. coli* F5 (K99) (IgG), and *S. uberis* (IgG and IgG1). The serum IgG contains statistically more antibodies to BRSV (IgG2), *S. aureus* (IgG), *S. uberis* (IgG2), and rotavirus (IgG and IgG2). Serum IgG is also higher in antibodies to BCV than milk IgG.

Assessment of the magnitude of differences in antibodies in IgG in colostrum, milk and serum:

To consider the potential efficacy of the three bovine IgG sources for passive transfer in newborns, the average values of ELISA units for each target antigen were calculated (Table 2).

To better illustrate the differences between the biologics, the doubling dilution estimates, and average ELISA unit values were used to estimate the magnitude of differences among the samples to each target and expressed as the magnitude of differences or fold change. For example, if the doubling dilution for the BRSV assay was estimated at 10 OD units from the standard curves, then the magnitude of the difference between milk and colostrum would be (248 ELISA units-221 ELISA units)/10 ELISA units ≥ 2-fold magnitude difference (Table 2).

Colostrum (IgG) antibodies specific to BRSV were 11-fold higher in the colostrum, compared to the serum (Table 2). For all other pathogen targets colostrum (IgG) antibodies were at least 1-fold higher than serum for all pathogens tested, except BCV, which was nearly identical in all three biologics, and *S. aureus*, which is greater than 8-fold higher in the serum. Colostral (IgG) antibodies were 1- to 3-fold higher than the milk for all pathogen targets except BHV-1. Magnitude differences between serum and the two lacteal secretions were even more marked for some targets, ranging from 1 to 11-fold higher antibody in the lacteals compared to serum. Most notably, colostrum and milk IgG providing greater than 8-fold higher antibodies to BRSV in comparison to the serum. *S. aureus* specific antibody was present in substantially higher levels in

the serum IgG than IgG of either lacteal secretion, 8-fold greater than colostrum and 13-fold greater than milk.

Table 2 also shows the calculated estimates of the magnitude of the differences for IgG1 and IgG2 subclass antibodies for a subset of pathogens. Subclass testing revealed that across all three targets, as expected, IgG2 differences were the most dramatic, with serum IgG containing much higher levels than IgG in lacteal fluids for BRSV (IgG2) and Rotavirus (IgG2). In particular, BRSV (IgG2) specific antibody is 16-fold higher in serum IgG, compared to colostrum IgG and 14-fold higher in serum IgG compared to milk IgG. In comparison to the other subclass testing, *S. uberis* (IgG2) specific antibodies are not as dramatically different in magnitude in the IgG across all three biologics, differing by 3-fold between serum and colostrum IgG and 4-fold between serum and milk IgG.

4.4 Discussion

The results of this study show that colostrum contained higher levels of antibodies per gram of IgG to BRSV, *S. uberis*, *E. coli* F5 (K99), and rotavirus than serum or milk. As anticipated, colostrum and milk IgG was predominately IgG1 subclass (Barrington, Besser, Davis, et al., 1997; Staley et al., 1972). Since only the IgG1 subclass antibody is re-secreted at mucosal surface (Besser 1988, Ellis et al., 2018), this is critical in disease protection in calves early in life.

Also as expected, serum had higher levels of IgG2 than lacteal samples to all targets tested, with the exception of *S. uberis* specific IgG2 that was highest in the colostrum. Antibodies (IgG) to *S. aureus* were highest in serum IgG. It is likely that this is due to increased IgG2 subclass production to this agent and its more abundant representation in the serum (reviewed in, Dego et al., 2002). However, in comparison to colostral IgG, serum IgG was much lower in antibodies to BRSV (IgG and IgG1), *S. uberis* (IgG), rotavirus (IgG1) and *E. coli* F5 (K99) (IgG).

Milk-derived IgG was lower in magnitude (per gram of IgG) compared to colostrum-derived IgG for most agents tested in this study. However, Milk IgG was superior to serum IgG, providing significantly higher specific antibody to BRSV (IgG and IgG1), *S. uberis* (IgG and IgG1), *E. coli* F5 (K99) (IgG) and rotavirus (IgG). Additionally, similar to colostrum, milk IgG skews toward the IgG1 subclass that is biologically important for the neonate.

The sample size of this study was small, with only 24 animals tested. However, in terms of total IgG and farm management practices, they were representative of a standard dairy herd Overall, the findings of this study, which was, to the best of our knowledge the first that attempted to estimate and compare the specificities of the antibodies sourced from colostrum, milk and serum, support the notion that colostrum is the superior source for protective antibodies for newborn calves. Nevertheless, our results indicated that milk also had significant levels of IgG1 immunoglobulins that are critical to protection from a number of ubiquitous calfhood pathogens, and could be used to supplement colostrum sourced IgG. However, milk immunoglobulins were tested from a single timepoint and are not representative of the entirety of a lactation cycle. As well, the number of animals tested in this experiment was small and larger sample size from multiple farms may be advisable to confirm and extend this finding.

Table 1: Serum total protein (TP) levels and IgG concentration, milk Brix and IgG concentration, and colostrum Brix and IgG concentration in 24 post partum dairy cows and heifers.

	IgG (g/L)			**TP (g/L)**	**Brix (%)**	
N=24	**Serum**	**Milk**	**Colostrum**	**Serum**	**Milk**	**Colostrum**
Average	24.3	1.2	94.6	9.2	10.8	23.8
Range	12.9-52.1	0.5-2.8	12.4-201.7	7.4-11.4	8.5-12.1	11.2-33.8

Figure 1a: ELISA unit values for antigen specific antibodies in IgG (H+L) from colostrum, milk and serum for 24 post partum dairy cows and heifers. Range of ELISA values is represented by boxes, points represent outliers and error bars represent standard deviation from the mean.

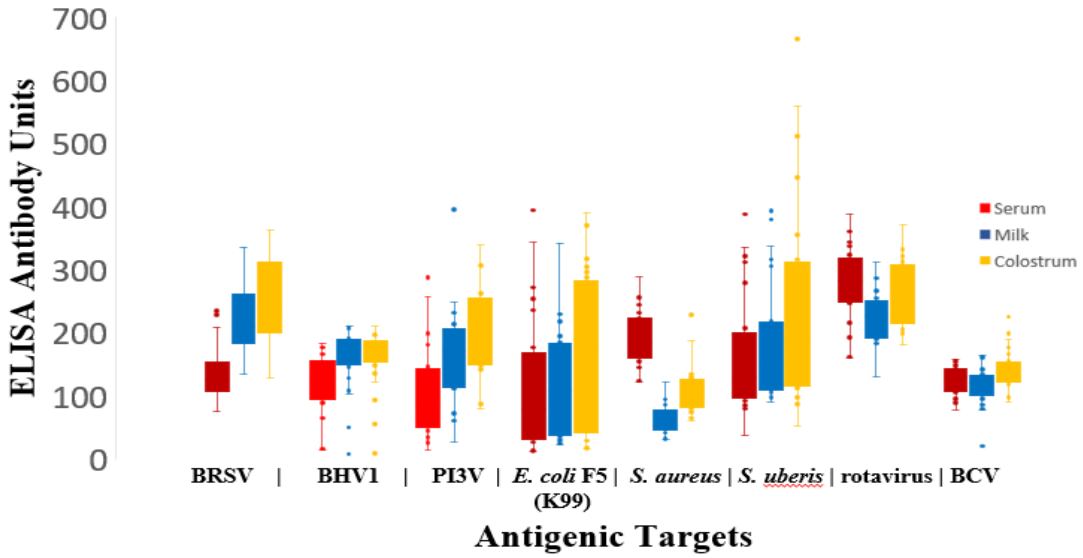


Figure 1b. ELISA unit values for antigen specific antibodies in IgG1 and IgG2 subclasses from colostrum, milk and serum for 24 post partum dairy cows and heifers. Range of ELISA values is represented by boxes, points represent outliers and error bars represent standard deviation from the mean.

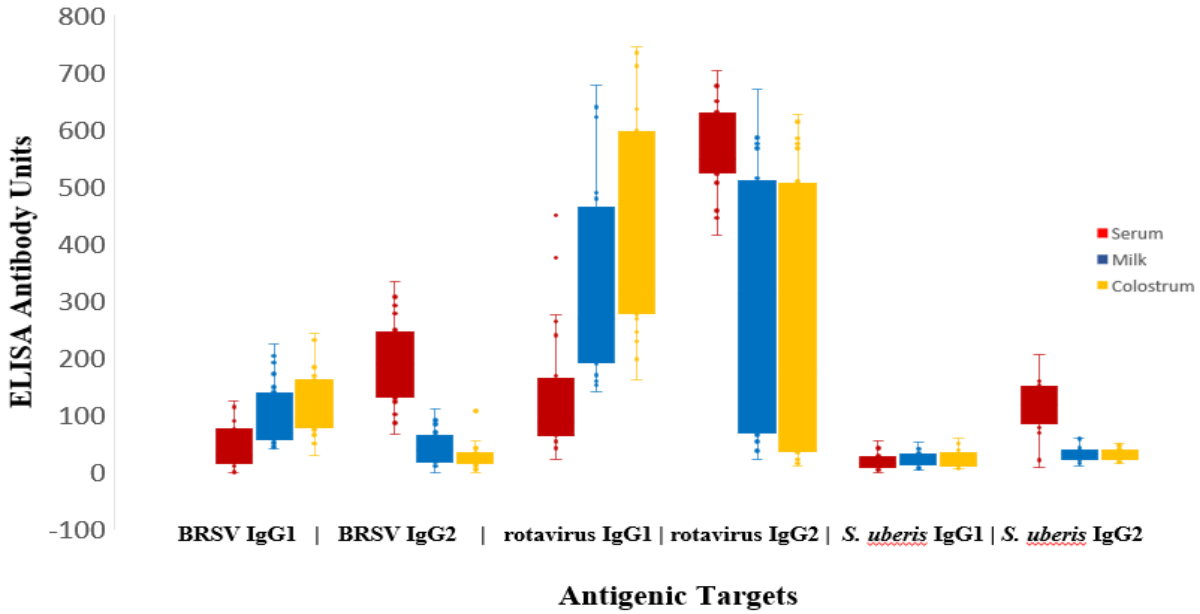
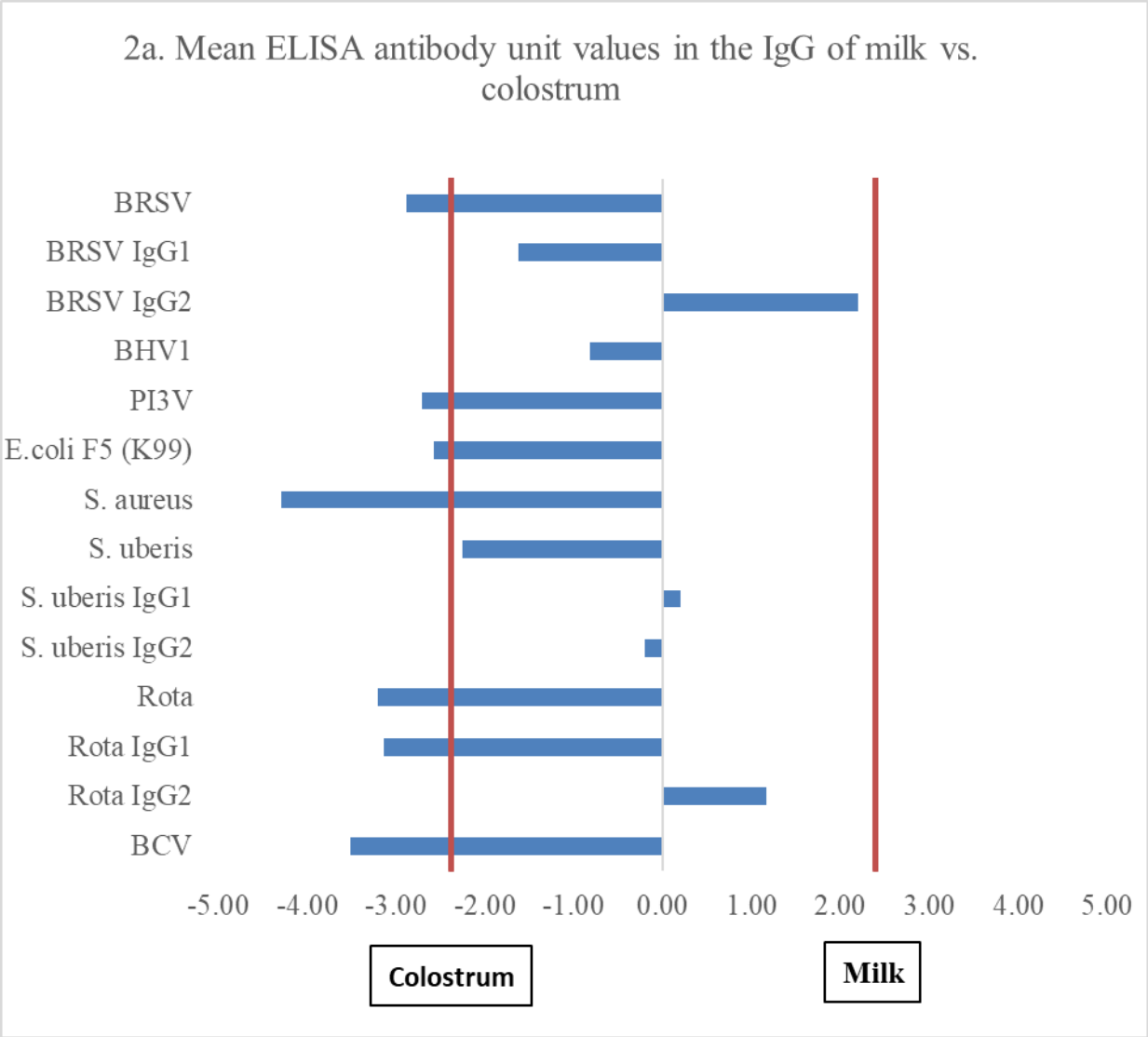
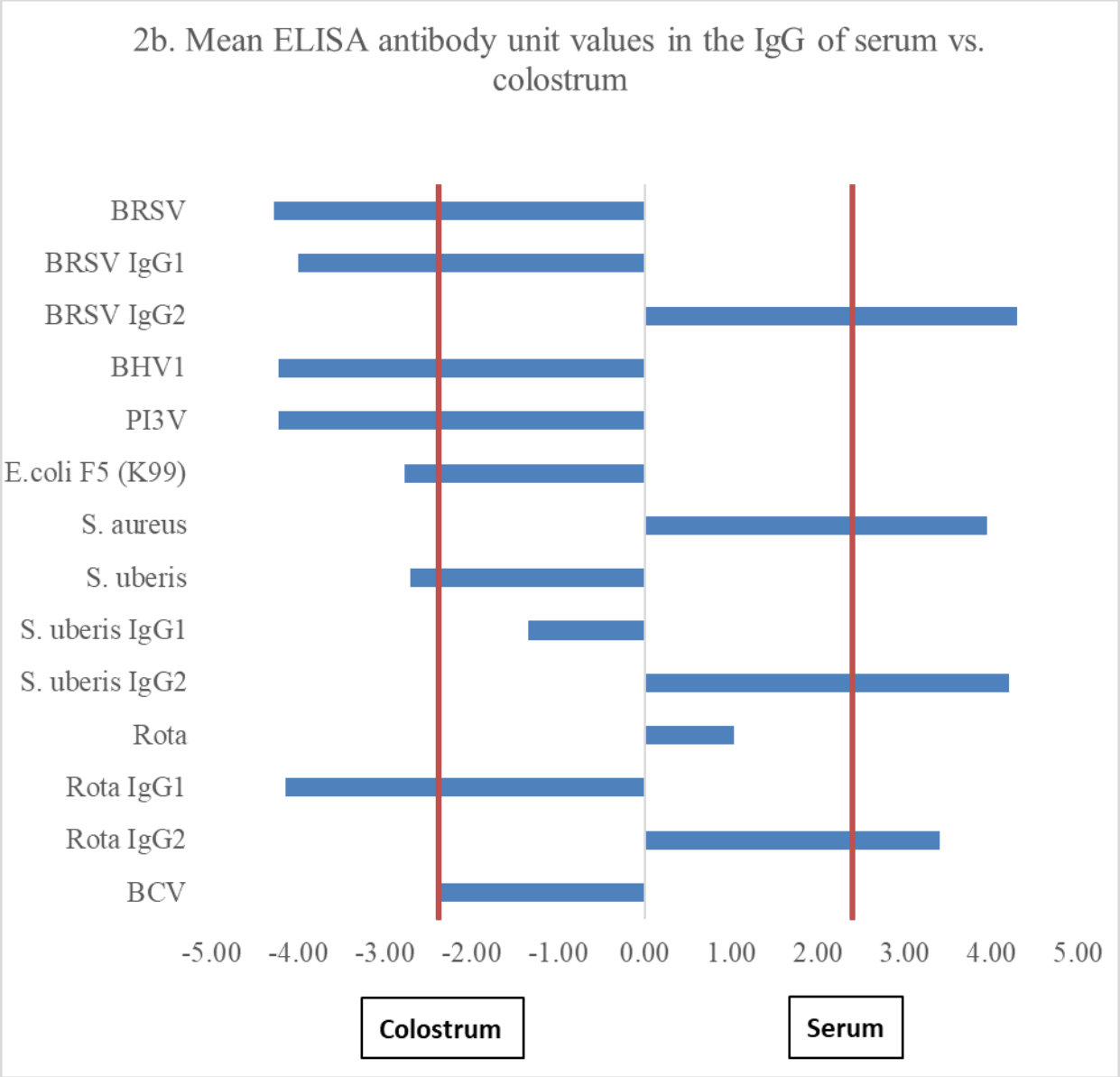


Figure 2: Comparison of mean ELISA units for antigen specific antibodies in IgG from colostrum, milk and serum in a group of 24 dairy cows and heifers. (Wilcoxon signed rank test with Bonferroni correction, P<0.017). Values greater than 2.385, or less than -2.385 (red line) denote statistically significant differences between the two biological samples being compared. Unless specified, the secondary antibody target is directed against the heavy and light chains (H+L) of IgG.




2b. Mean ELISA antibody unit values in the IgG of serum vs. colostrum
BRSV
BRSV IgG1
BRSV IgG2
BHV1
PI3V
E.coli F5 (K99)
S. aureus
S. uberis
S. uberis IgG1
S. uberis IgG2
Rota
Rota IgG1
Rota IgG2
BCV
-5.00
-4.00
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
4.00
5.00
Colostrum
Serum

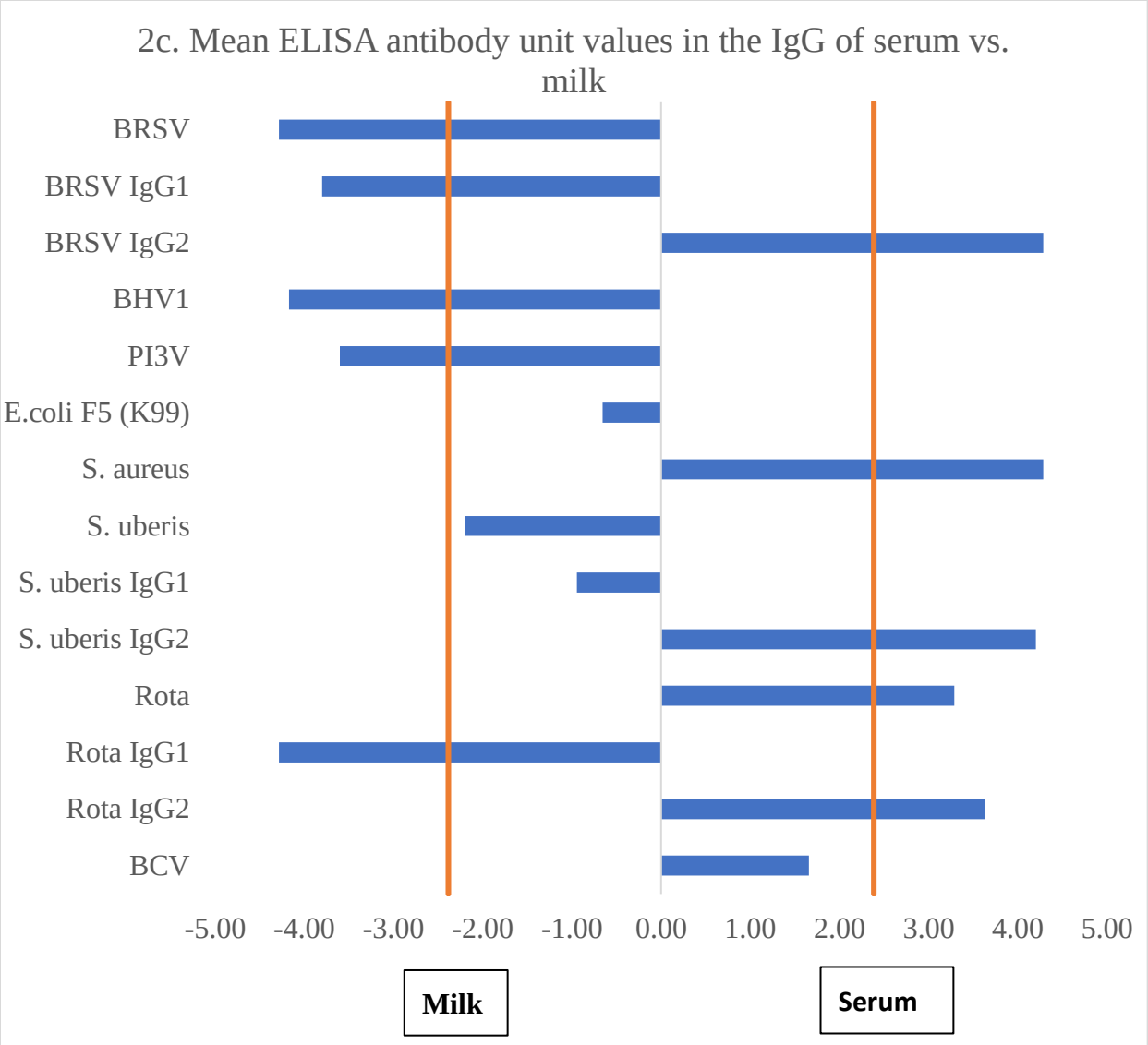

2c. Mean ELISA antibody unit values in the IgG of serum vs. milk
BRSV
BRSV IgG1
BRSV IgG2
BHV1
PI3V
E.coli F5 (K99)
S. aureus
S. uberis
S. uberis IgG1
S. uberis IgG2
Rota
Rota IgG1
Rota IgG2
BCV
-5.00
-4.00
-3.00
-2.00
-1.00
0.00
1.00
2.00
3.00
4.00
5.00
Milk
Serum

Table 2. Mean ELISA antibody units in the IgG of the colostrum, milk and serum for each target antigen in a group of 24 dairy animals, the number of ELISA units approximately equal to a doubling of the amount of antibody and an estimate of the magnitude (doubling) and direction of the differences in ELISA units in antibodies in IgG from colostrum, milk and serum*.

	Average ELISA units				**Approx. magnitude of differences in average ELISA units**		
Specific antibody target antigen	**Serum IgG**	**Milk IgG**	**Colostrum IgG**	**ELISA Units = 1 dbling dilution in assay***	**Colostrum vs serum IgG**	**Milk vs serum IgG**	**Colostrum vs milk IgG**
BRSV H+L	**137**	**221**	**248**	**10**	**>11**	**>8**	**>2**
BRSV IgG1	**42**	**99**	**115**	**10**	**>7**	**>5**	**>1**
BRSV IgG2	**192**	**43**	**28**	**10**	**<-16**	**<-14**	**<-1**
BHV-1 H+L	**124**	**159**	**162**	**15**	**>2**	**>2**	**>0**
PI3V H+L	**106**	**164**	**201**	**30**	**>3**	**>1**	**>1**
***E. coli* F5 (K99) H+L**	**118**	**120**	**174**	**20**	**>2**	**>0**	**>2**
***S. aureus* H+L**	**194**	**62**	**109**	**10**	**<-8**	**<-13**	**>4**
***S. uberis* H+L**	**163**	**189**	**244**	**25**	**>3**	**>1**	**>2**
***S. uberis* IgG1**	**20**	**23**	**23**	**25**	**>0**	**>0**	**>0**
***S. uberis* IgG2**	**117**	**32**	**31**	**25**	**<-3**	**<-4**	**<0**
Rotavirus H+L	**279**	**227**	**268**	**20**	**<0**	**<-2**	**>2**
Rotavirus IgG1	**134**	**324**	**398**	**20**	**>13**	**>9**	**>3**
Rotavirus IgG2	**561**	**298**	**269**	**20**	**<-14**	**<-13**	**<-1**
BCV H+L	**124**	**114**	**141**	**60**	**>0**	**<0**	**>1**

*Values obtained from assessment of serial dilutions of a positive control serum.

5. GENERAL DISCUSSION AND CONCLUSIONS:

Failure of passive transfer of maternal immunoglobulin (FPTI) is the primary factor contributing to morbidity and mortality of calves in commercial dairies (Windeyer et al., 2014). Commercial colostrum replacer products offer both dairy and beef producers a convenient source of nutrition and means to prevent FPTI. This study has examined the IgG in colostrum, milk and serum to determine if there are differences in the antibody specificity to better understand if immunoglobulin from these sources should be used interchangeably for facilitating passive transfer in newborn calves.

Dairy colostrum is the obvious source of immunoglobulin for CR products but is of limited volume and thus expensive and difficult to source. Serum antibodies have been utilized as an alternative immunoglobulin source in some countries. However, serum antibodies do not meet regulatory requirements in many jurisdictions and while serum can provide high concentrations of total Igs, the proportions of antibodies with IgG1 and IgG2 subclass differs from that of colostrum, which raises questions around the protective re-secretion properties of about 50% of serum sourced immunoglobulin (Besser 1988, Arthington et al., 2002).

Milk is another readily available source of immunoglobulin for CR products. Like colostrum (and unlike serum), milk Ig is primarily IgG1 (reviewed in, Tizard, 2018), critical to protection provided by re-secretion at mucosal surfaces (Besser & Osborn, 1993; Ellis et al., 2018). However, the specificity of antibodies in milk may differ from colostrum in which the IgG is primarily serum derived. While some antibodies in milk are derived via translocation from serum, 70% of IgG in the milk is produced locally, in the udder (reviewed in, Hurley, 2003). So, while the predominant subclass of immunoglobulin in milk and colostrum (IgG1 subclass) the source differs and that could affect the spectrum of specificities in each secretion. Thus, it is pertinent to understand if the antibody specificities from these lacteal secretions differ significantly.

Currently, there are no previous studies addressing the question of the specificity of immunoglobulins in milk whey (or serum) compared to the IgG in colostrum. A more complete understanding of the antibody specificity could influence their use and potentially lead to improved CR products and improved calf health and performance outcomes.

A prerequisite for this study was a reliable means to quantify the levels of antibodies recognizing the target antigens. The qualitative ELISA is primarily an indicator of the presence, or absence of a target. At best, it is an ordinal measure of antibody concentration (Tyler & Cullor, 1989). In a qualitative indirect ELISA, positive samples may need to be diluted by many orders of magnitude before the magnitude of the value changes to demonstrate differences in antibody level between samples in the same test. Generally speaking, the qualitative ELISA can only give an indication of "high", "medium" and "low" levels of antibody. This is because the samples are read at a single dilution and compared to a positive control that has been titrated to determine the standard curve for the assay (Durham & Sillars, 1986; Graham et al., 1998). The positive control is read at the top of the linear portion of the standard curve, and the sample dilutions ideally fall within the linear portion of this curve. For the purposes of this study, it was important to compare the amount of each specific antibody per gram of IgG directly between the serum, milk, and colostrum samples more precisely. Thus, there was a need to modify the existing ELISAs for increased precision. As shown in Table 1 the samples tested varied by up to 100X in IgG concentration. To improve precision, the IgG was adjusted to the same concentration for all samples to help ensure that the values fell within the linear range of the standard curve. In addition, for many of the assays used in this study, the slope of the standard curve was very shallow, and varied among target antigens making it difficult to understand how differences in units related to actual differences in magnitude of specific antibodies (Appendix A). That is, a steeper standard curve results in a smaller OD change resulting from one doubling dilution (a doubling dilution is a change of antibodies of one-fold), while the converse is true for a shallower standard curve where there was a greater change in OD equivalent to a doubling dilution. For example, for the BRSV (H+L) assay, a change in OD of 10 units is approximately equivalent to a doubling dilution, while the much shallower standard curve of a PI3 titration results in 30 OD units for a doubling dilution. Therefore, a doubling dilution (which is a fold difference in antibodies) was not equivalent to double the units. We attempted to account for the slope of the standard curves by estimating the magnitude of change of units that would equate to one doubling dilution of the antibodies in the assay (Table 2) and thus enable an estimate of the fold differences in antibodies among samples.

We tested serum, milk and colostrum of 24 dairy cattle from a single dairy farm in New York State for antibody specificities to the IgG of eight common pathogens of dairy cattle involved in either mastitis (*S. aureus* and *S. uberis*), NCD (BCV, rotavirus, *E. coli F5 (K99)*) or BRD (PI3,

BRSV, BHV-1, BCV). We further tested the IgG1 and 2 subclasses antibodies for one pathogen from each category (BRSV, *S. uberis*, rotavirus). The results of the specific antigen ELISAs on individual animals show a high degree of variability between each biologic for each target (Appendix B), even when all samples are tested at the same standardized total IgG value. While the strategy described previously allowed a comparison of the amounts of antibodies in different samples within each assay there was no means to compare between different assays. Many factors such as natural variations in the binding affinity and avidity of the antigen-antibody complex, antigenicity of agent and natural changes in cell culture can affect the sample dilution selected, as well as the slope and range of the standard curve. Physiological differences in individual cows and even environmental exposures and vaccination composition can influence background binding of each assay. Thus, the values obtained in an IgG normalized, semi-quantitative ELISA for BRSV using colostrum can be compared to the numbers obtained from testing serum or milk from this or other animals but cannot be compared to an assay for another target, such as rotavirus in either this or other animals.

We also tested for specific antibodies to the same pathogens in the IgG of forty-one commercially available products. These data are presented in Appendix C. For some products (n=12) it was not possible to discern the source of the IgG, for others the products appeared to be formulas with "mixed" IgG sourced from serum as well as either colostrum or whey (n=7). The results of testing the products of known Ig source, overall, support the findings of the study of the individual animal testing. Even without consideration of the differences in IgG subtypes, milk and colostrum Ig based products were more similar in most specific antibodies than products sourced from serum.

Limitations of the study and future directions:

The sample size of this study was small, with only 24 animals tested. However, in terms of total IgG and farm management practices, they were representative of a standard dairy herd. To add statistical weight to the testing in this study, it would be beneficial to apply the methodology used here to test serum, milk and colostrum samples from a much larger group of more geographically distributed animals. As well, the milk samples tested were sampled at a single timepoint and are not representative of the Ig profile over the entire lactation cycle. In addition,

there is merit to testing the commercially available colostrum products to determine the differences in specific antibodies among products. In the current study a variety of products, mainly from the US market (as Canada has only colostrum-based products) were tested and these data are shown in Appendix C. A significant limitation to these data is that most products sold in US markets are unlicensed and therefore ingredients are unknown.

Indirect ELISAs, even with the modifications used in this study are not precisely quantitative and therefore the data is only an estimate of the differences in levels of specific antibodies. More precise assays would be desirable but are not currently readily available. Additionally, the indirect ELISA, demonstrates antibodies binding to the infectious agent, and those antibodies may bind portions of the agent that are not essential for viral invasion of host cells, and/or other means of pathogenic effects, i.e., these ELISAs are a measure of many protein epitopes, both neutralizing and non-neutralizing, and not necessarily an indication of the presence or scale of pathogen neutralizing antibody. Antibody levels are not necessarily predictive of disease (sometimes calves with high Ab levels experience morbidity, sometimes calves with low antibody levels appear protected). Purification of ELISA antigens to create targets to highly immunogenic proteins receptors may provide more robust answers regarding the nature of specific immunoglobulins to each target agent, while broadening the range of the standard curve and giving us more comparative strength in regard to the antibody levels at the high and low end of these assays.

Feeding trials in newborn calves are the ultimate means to test the efficacy of these products. Trials comparing colostral and serum based CR products have had variable success (reviewed in, Cabral et al., 2013; reviewed in, Godden & James, 2020) leading to questions around the effects of processing colostral and serum antibodies on functionality and absorptive efficiencies. While the specific antibody profile of milk closely resembles that of colostrum, the magnitude of antibody in this biologic both in terms of overall IgG concentration and of specific antibodies of relevance per gram of IgG is significantly lower. Additionally, if milk whey used for CR production is collected from cheese production waste, it must be considered that this byproduct has undergone additional processing (e.g., rennet addition) and pasteurization without consideration for the need to preserve antibody functionality, so additional testing and modifications would need to be considered and tested. So while milk-derived IgG appears to have

utility, whey-derived products should be tested in controlled feeding trials for functionality and absorptive efficiencies in comparison to maternal colostrum and commercial counterparts.

Testing of the currently available colostrum replacer products was limited by difficulties in accessing information about the source of the IgG in many of the products. In Veterinary Biologics licensed products the source is colostrum however there is no means to know the source in unlicensed products, which are the vast majority available commercially. This is further complicated in that the Ig in many unlicensed products changes with time and cost of obtaining IgG from different sources.

Conclusions:

Colostrum ingestion and the resultant passive transfer of specific antibodies is a prerequisite for calf health. Colostrum replacement products have a role in ensuring optimal passive transfer management. Understanding of composition and efficacy of CR product IgG will lead to better quality products able to inform management decisions around calf health and lead to better disease management outcomes.

Serum-based products are not currently used in Canada, but regulations in many other countries including the USA allow commercial CR products comprised in whole or in part of bovine-serum Igs. In this small group of animals, compared to colostrum, serum appears to provide increased levels of antibodies (per gram of IgG) to *S. aureus* and if additional agents are tested may prove to have other, targeted benefit over the lacteal biologics to specific pathogens. However, overall, serum IgG has lower levels of antibodies to most pathogens of importance for newborn calves and the benefit of serum Igs for CR products further is reduced by the large amount of IgG2 subclass present (about 50%), diminishing the magnitude of protective re-secretion at mucosal surfaces of the calves (Besser 1988, Ellis 2019).

The results of this study indicate that milk sourced IgG is a more promising ingredient to supplement the scarce supply of Ig available from colostrum for day one use for passive transfer. In contrast to serum-based IgG the antibodies found in milk are more similar in specificity (per gram of IgG) and subclass distribution (predominately IgG1) to those found in colostrum.

In conclusion, the results of this study suggest that, when directly comparing serum, milk and colostrum IgG for the antibodies to target pathogens, colostrum is the best source of immunoglobulin to use in colostrum replacer products. However, given the similar antibody subtype profile and specificity of milk immunoglobulin, harvesting, purifying and concentrating milk Igs could also be an acceptable alternative for commercial CR products.

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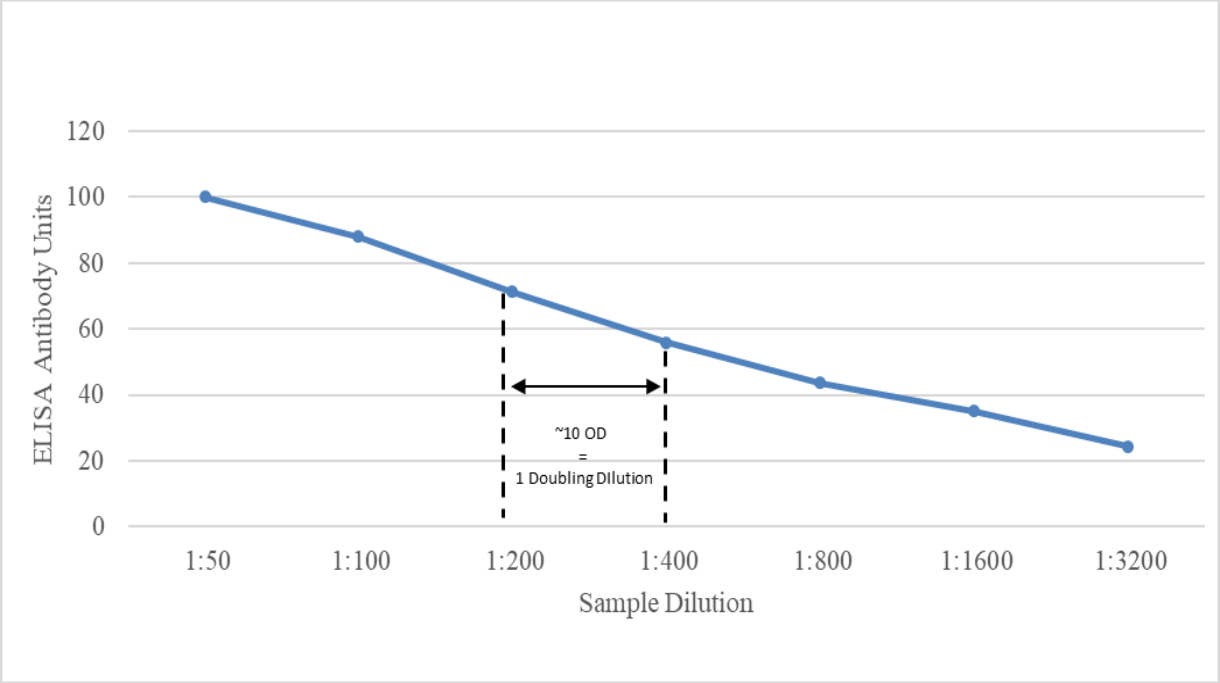
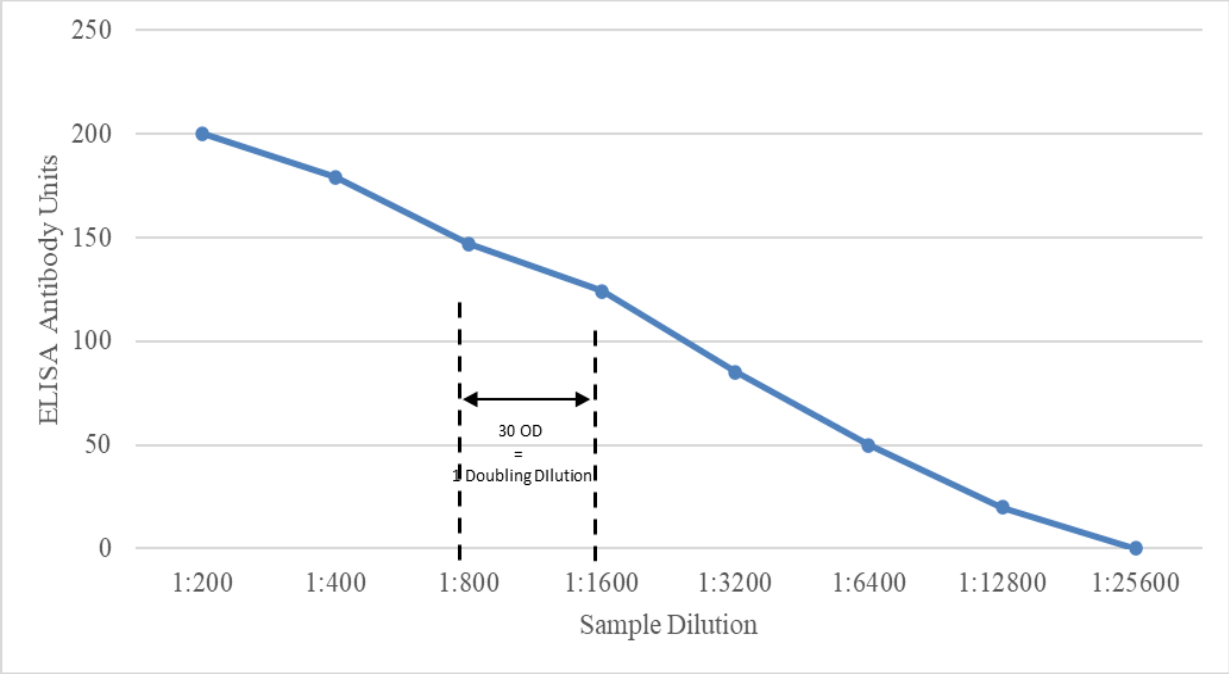
APPENDIX

A. Titrations of Positive Control Serum to Determine ELISA-Target Specific Magnitude Changes in ELISA Units.

A. 1. Two-fold serial dilution of BRSV positive control serum tested in an indirect ELISA to determine magnitude of optical density unit change that equates to one doubling dilution of the sample in the assay.



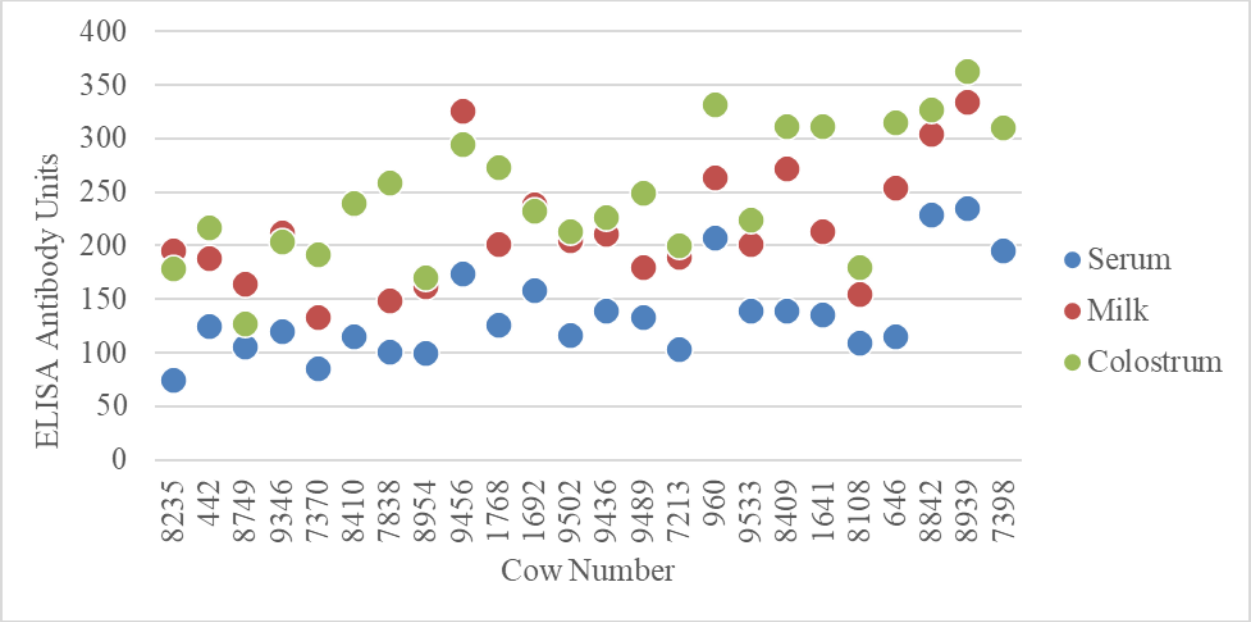
A.2. Two-fold serial dilution of PI3V positive control serum tested in an indirect ELISA to determine the magnitude of optical density units change that equates to one doubling dilution in the assay.



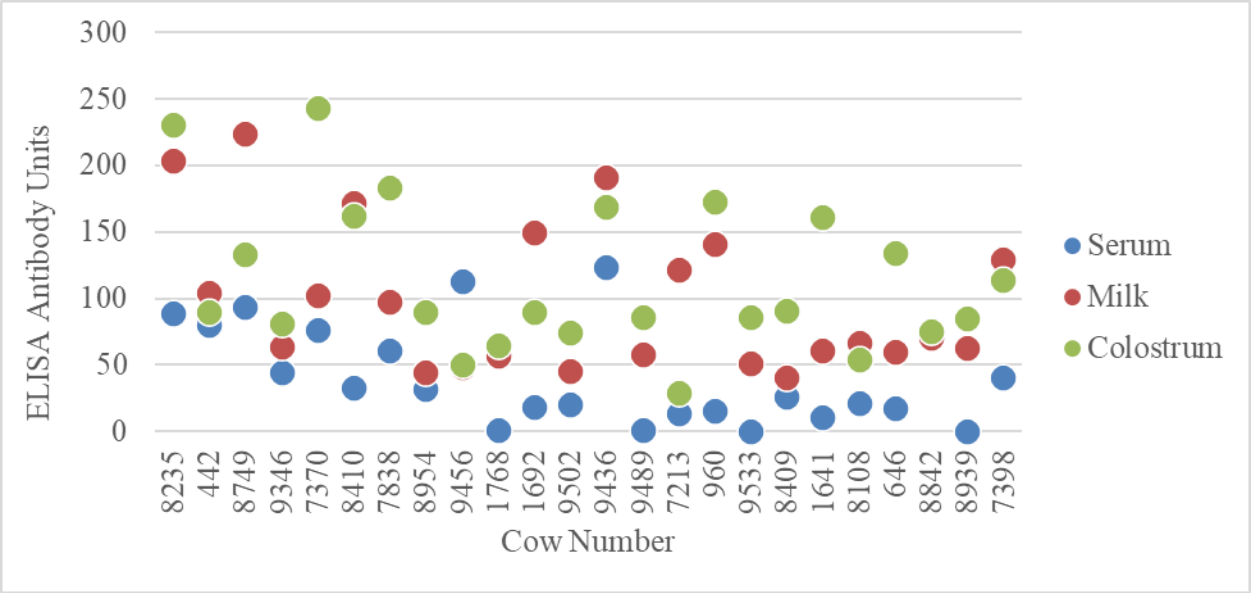
B. Individual Cow or Heifer Specific Antibody ELISA Units

ELISA unit values for antigen specific antibodies in IgG from colostrum, milk and serum (a-n) for 24 post partum dairy cows and heifers.

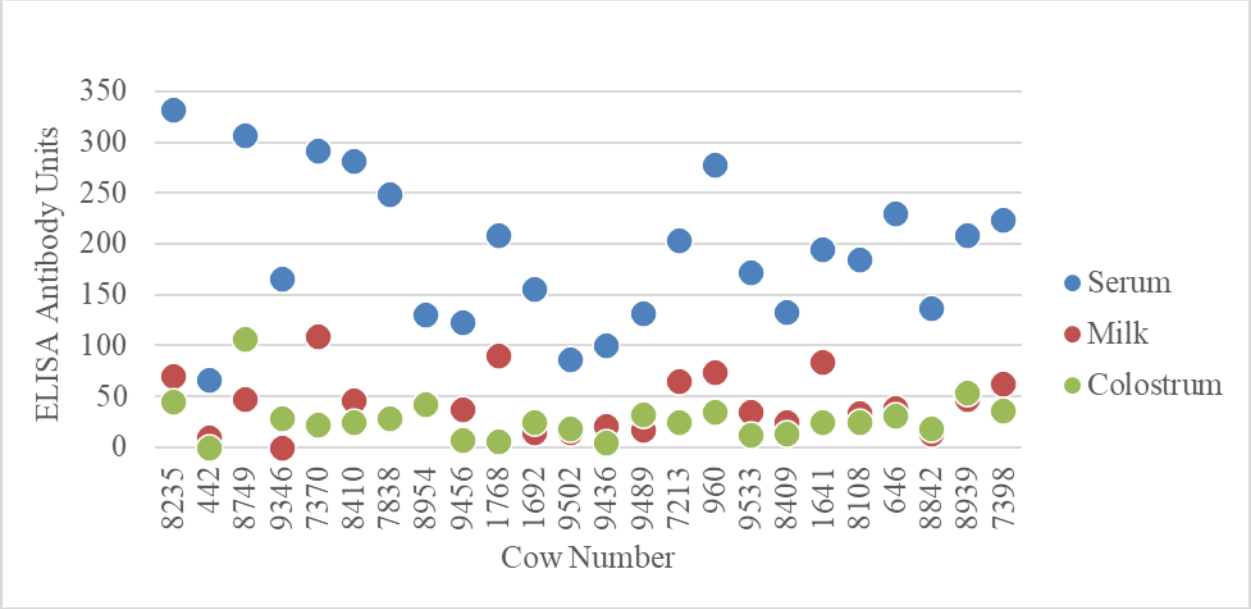
B.1. BRSV specific IgG (H&L) ELISA antibody units in the IgG of serum, milk and colostrum for 24 post partum dairy cows and heifers.



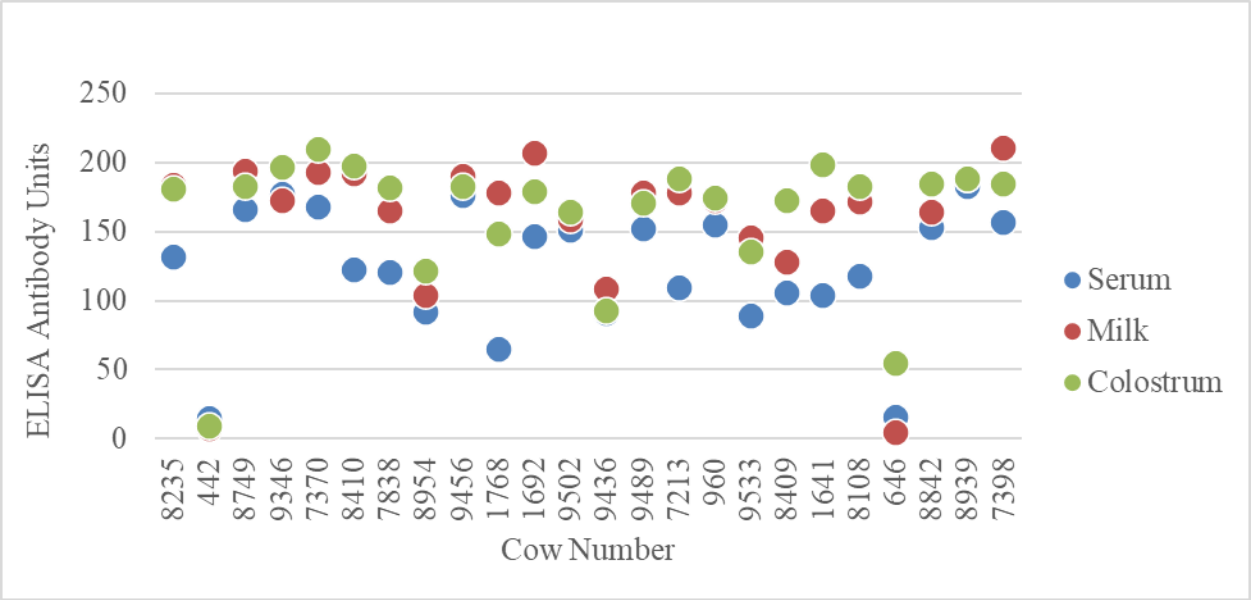
B.2. BRSV specific IgG (IgG1) ELISA antibody units in the IgG of serum, milk and colostrum for 24 post partum dairy cows and heifers.



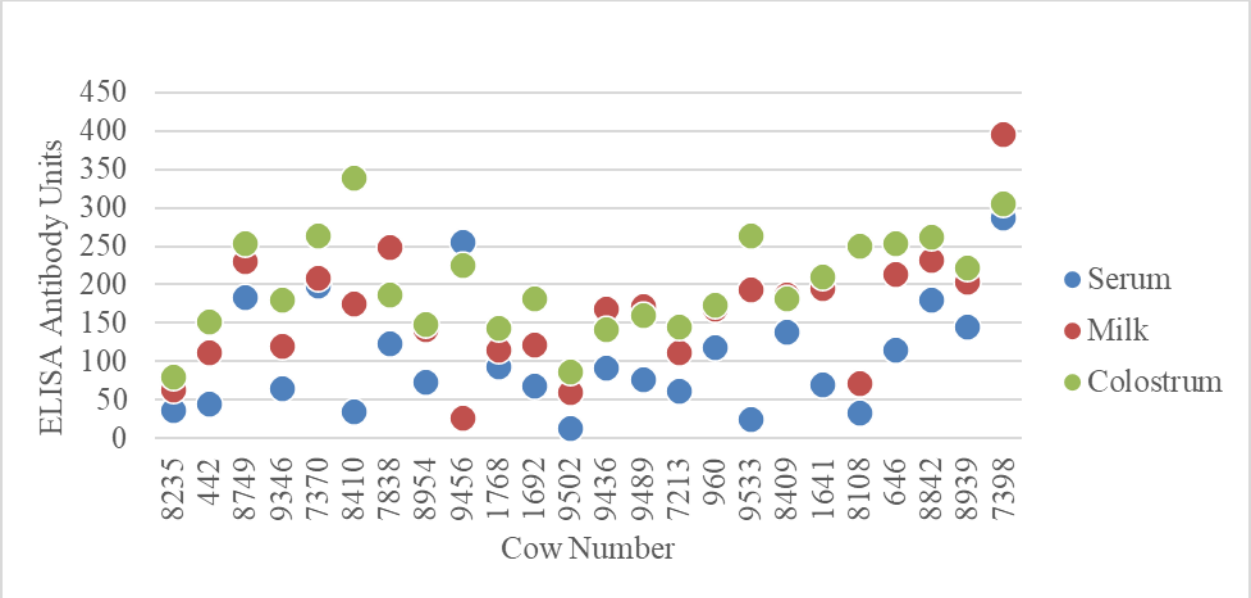
B.3. BRSV specific IgG (IgG2) ELISA antibody units in the IgG of serum, milk and colostrum for 24 post partum dairy cows and heifers.



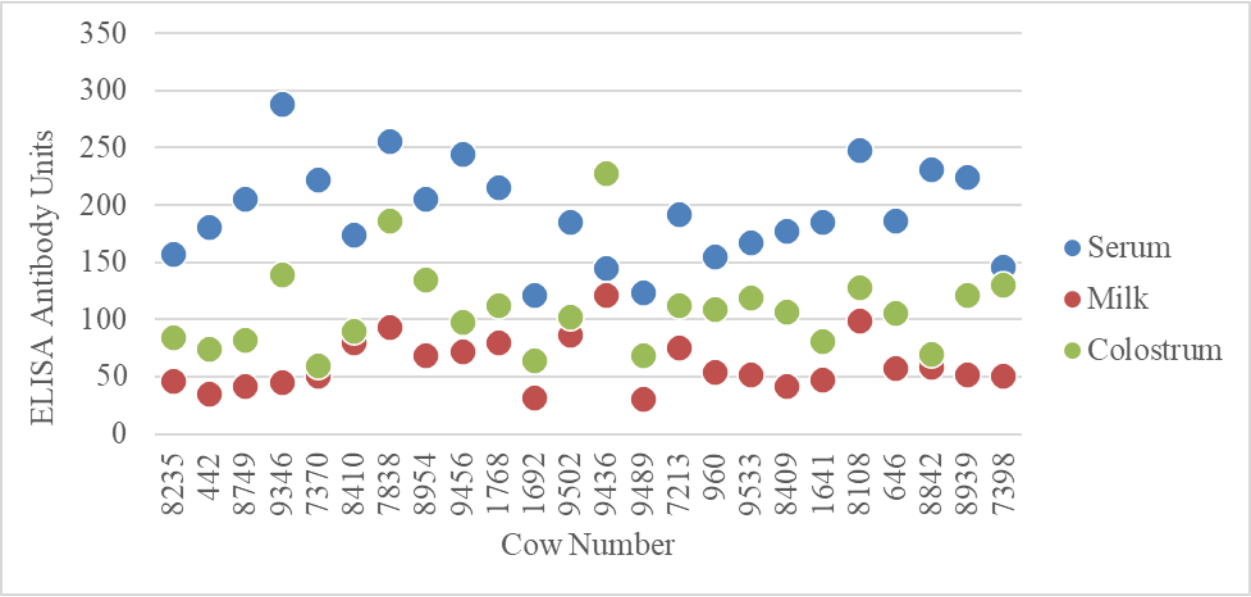
B.4. BHV-1 specific IgG (H&L) ELISA antibody units in the IgG of serum, milk and colostrum for 24 post partum dairy cows and heifers.



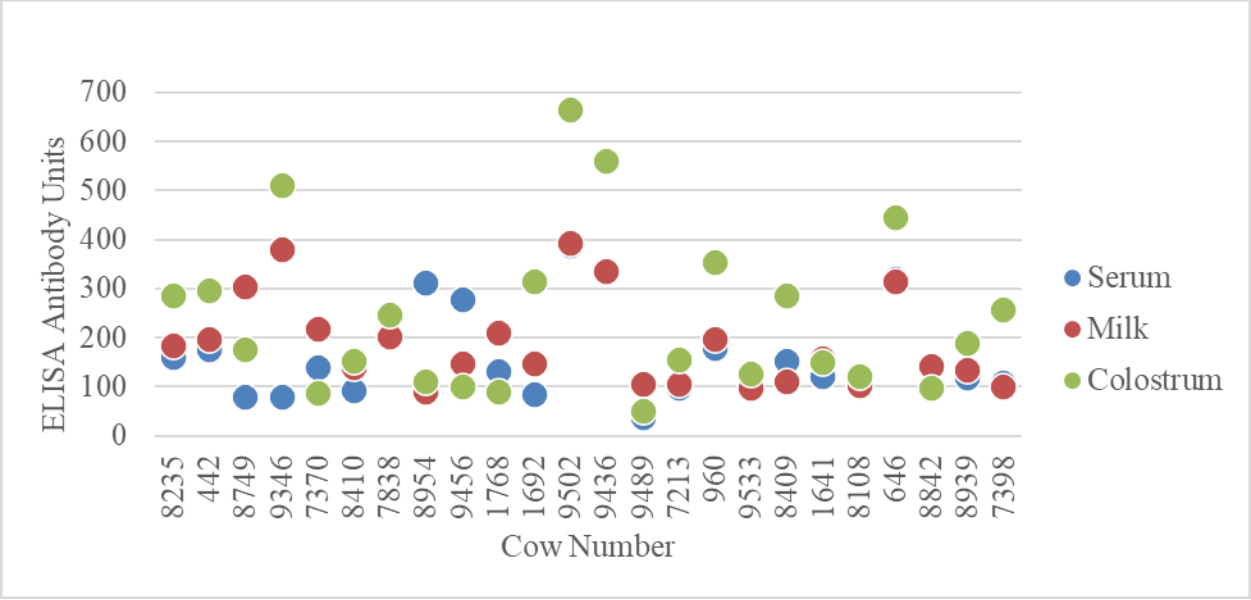
B.5. PI3V specific IgG (H&L) ELISA antibody units in the IgG of serum, milk and colostrum for 24 post partum dairy cows and heifers.



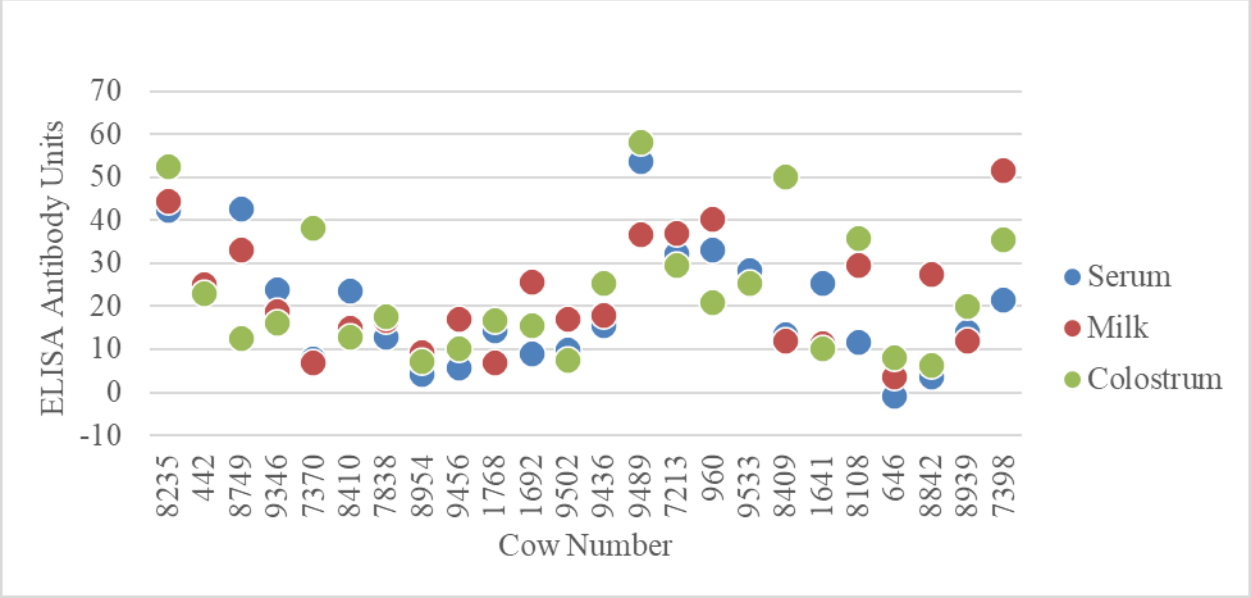
B.6. *S. aureus* specific IgG (H&L) ELISA antibody units in the IgG of serum, milk and colostrum for 24 post partum dairy cows and heifers.



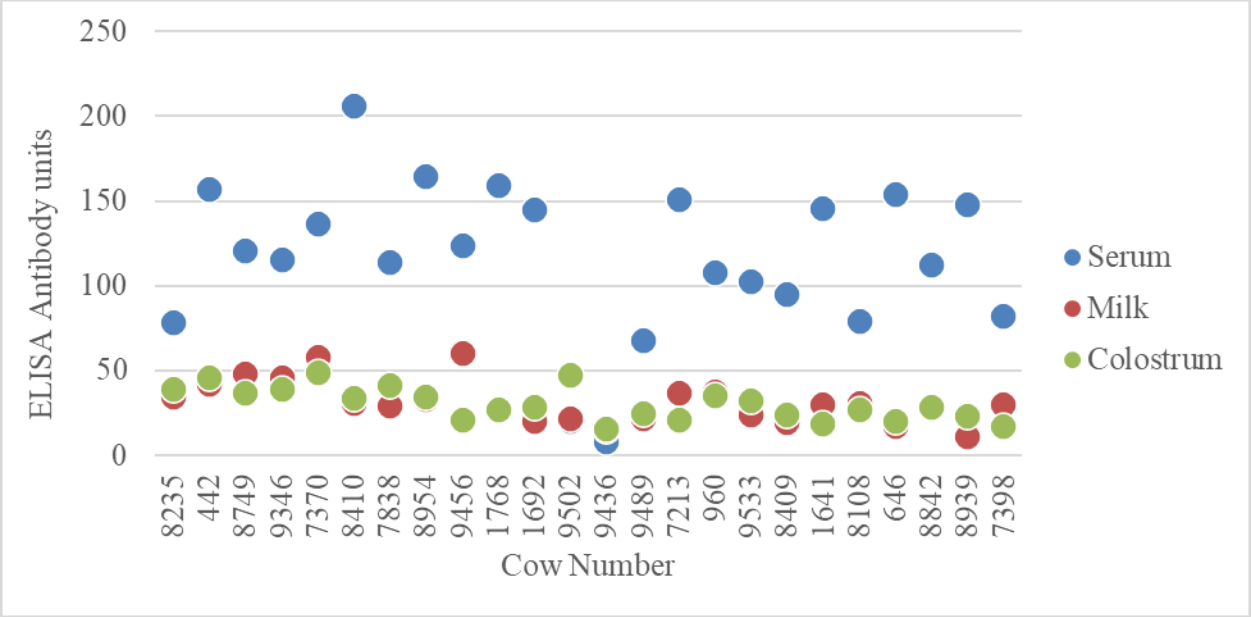
B.7. *S. uberis* specific IgG (H&L) ELISA antibody units in the IgG of serum, milk and colostrum for 24 post partum dairy cows and heifers.



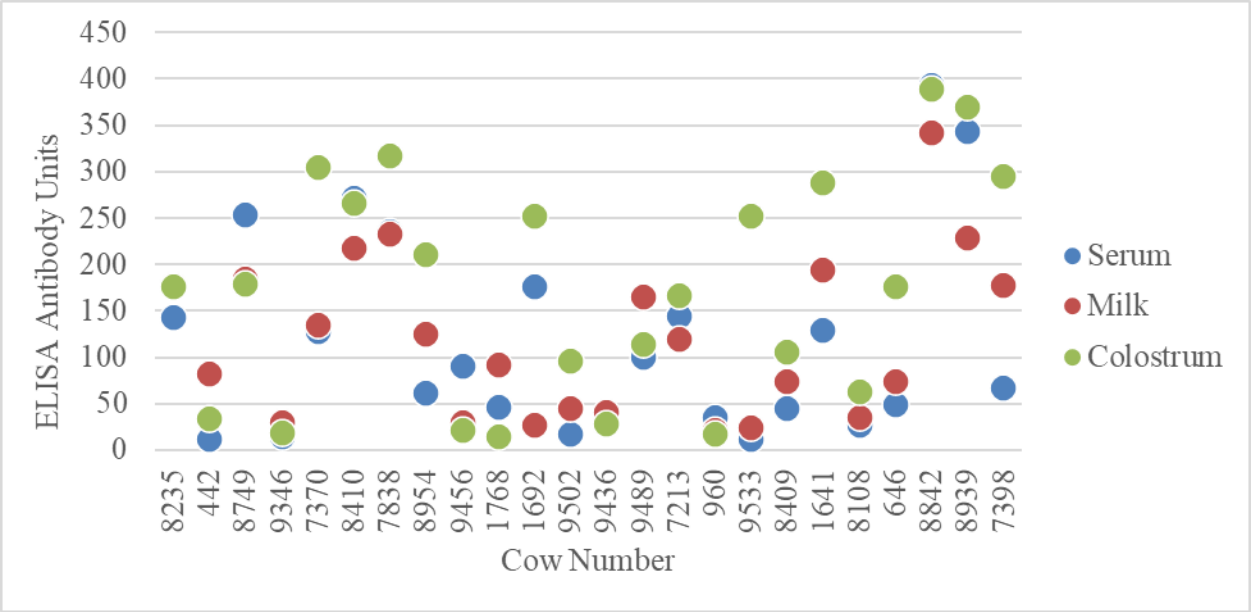
B.8. *S. uberis* specific IgG (IgG1) ELISA antibody units in the serum, milk and colostrum for 24 post partum dairy cows and heifers.



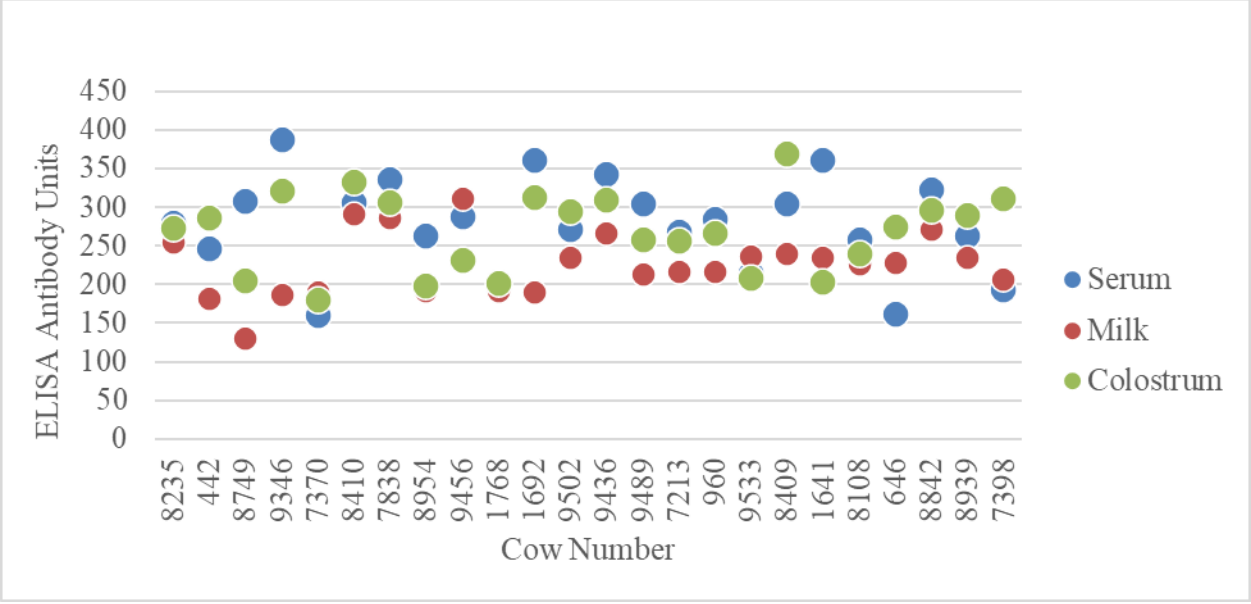
B.9. *S. uberis* specific IgG (IgG2) ELISA antibody units in the serum, milk and colostrum for 24 post partum dairy cows and heifers.



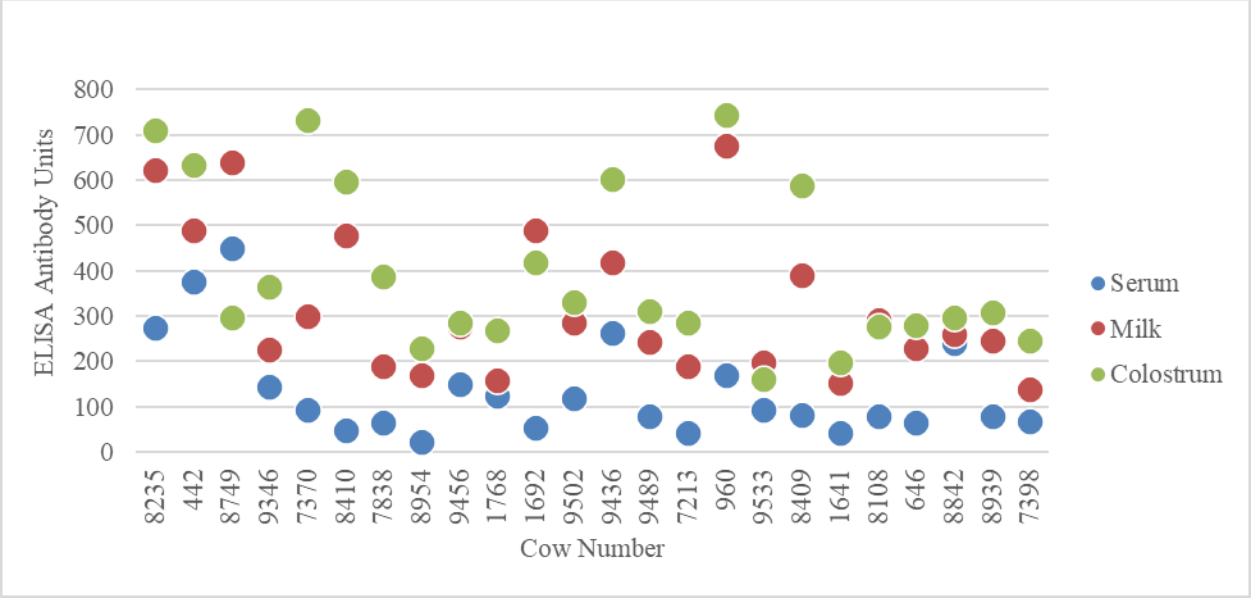
B.10. *E. coli* F5 (K99) specific IgG (H&L) ELISA antibody units in the IgG of serum, milk and colostrum for 24 post partum dairy cows and heifers.



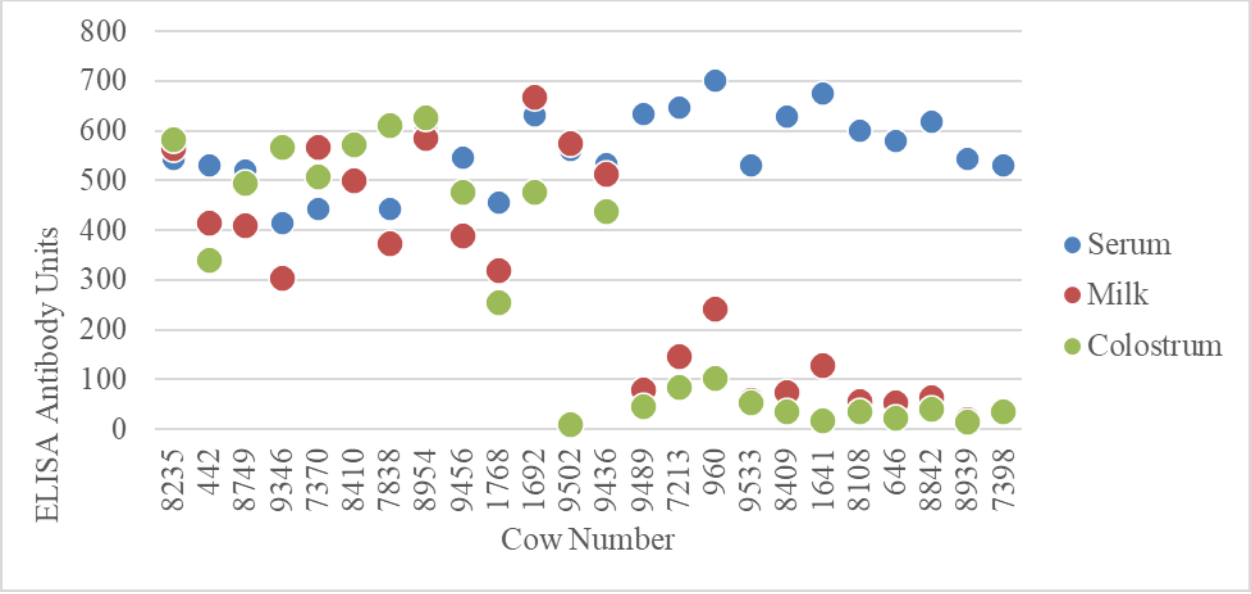
B.11. Rotavirus specific IgG (H&L) ELISA antibody units in the IgG of serum, milk and colostrum for 24 post partum dairy cows and heifers.



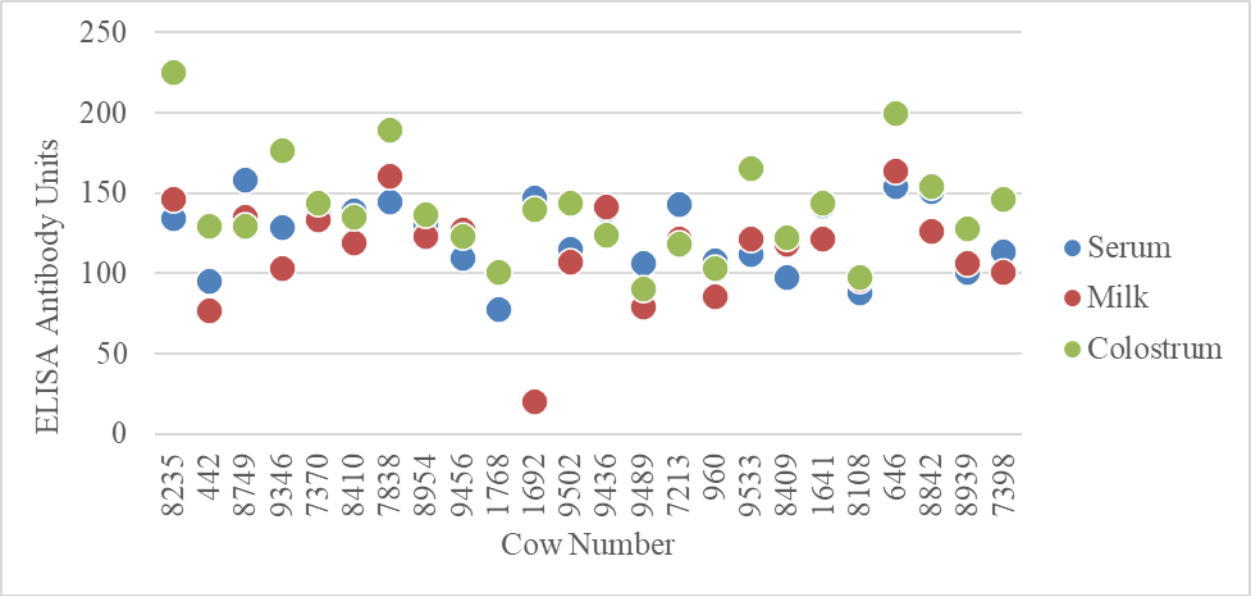
B.12. Rotavirus specific IgG (IgG1) ELISA antibody units in the serum, milk and colostrum for 24 post partum dairy cows and heifers.



B.13. Rotavirus specific IgG (IgG2) ELISA antibody units in the serum, milk and colostrum for 24 post partum dairy cows and heifers.



B.14. BCV specific IgG (H&L) ELISA antibody units in the IgG of colostrum, milk and serum for 24 post partum dairy cows and heifers.



C. Commercial Colostrum Replacer Product Testing

An attempt was made to assess differences among the specific antibodies present in commercially available colostrum replacer and feed products. Forty-one CR products were purchased from feed stores in North America. When possible, products were divided into groups based on their stated Ig source (see Appendix C). Products were reconstituted according to package directions. Similar to the testing protocol for samples from the cows and heifers used in this study, products were first tested for total IgG concentration via RID. This value was used to dilute each product to 0.5 g/L for specific target ELISA testing. As with the biologic sample testing, samples of these products were then further diluted, to working dilutions such that the values were expected to be within the linear portion of each of the ELISA standard curves. The values were normalized post-testing to reflect specific antibody amount per 1g/L total IgG. Product testing ELISAs were performed for IgG H+L only.

As shown in Appendix C, product testing revealed highly variable levels of specific antibodies in the IgG among products. Even within the products that claim to source Igs from maternal colostrum, total IgG was not indicative of levels of specific antibody per gram of IgG

across the targets tested. Many products, regardless of Ig source, had IgG with low specific antibodies to both PI3V and *E. coli* F5 (K99).

However, similar to the animal testing performed for this study, colostrum-derived product IgG generally were the highest in terms of levels of specific antibodies. These products were the highest of the other single-Ig-sourced products for BRSV, BHV-1 and PI3V specific antibodies and the other targets tested were generally high for specific antibodies in the colostrum CR products. Serum-based product IgG was lower in specific antibodies to BRSV, BHV-1 and PI3 than the milk and colostrum-derived products and highest for specific antibodies in *S. aureus, S. uberis*, and rotavirus (Appendix B & C). CR products made from milk whey would likely represent a broader spectrum of the lactation cycle while this study chose to analyze samples from a single timepoint (day 5). Despite being collected at a single timepoint, milk or whey-based product IgG was higher than serum IgG for BRSV, PI3V and BHV-1, and were generally comparable to the colostrum in regard to levels of the other targets tested.

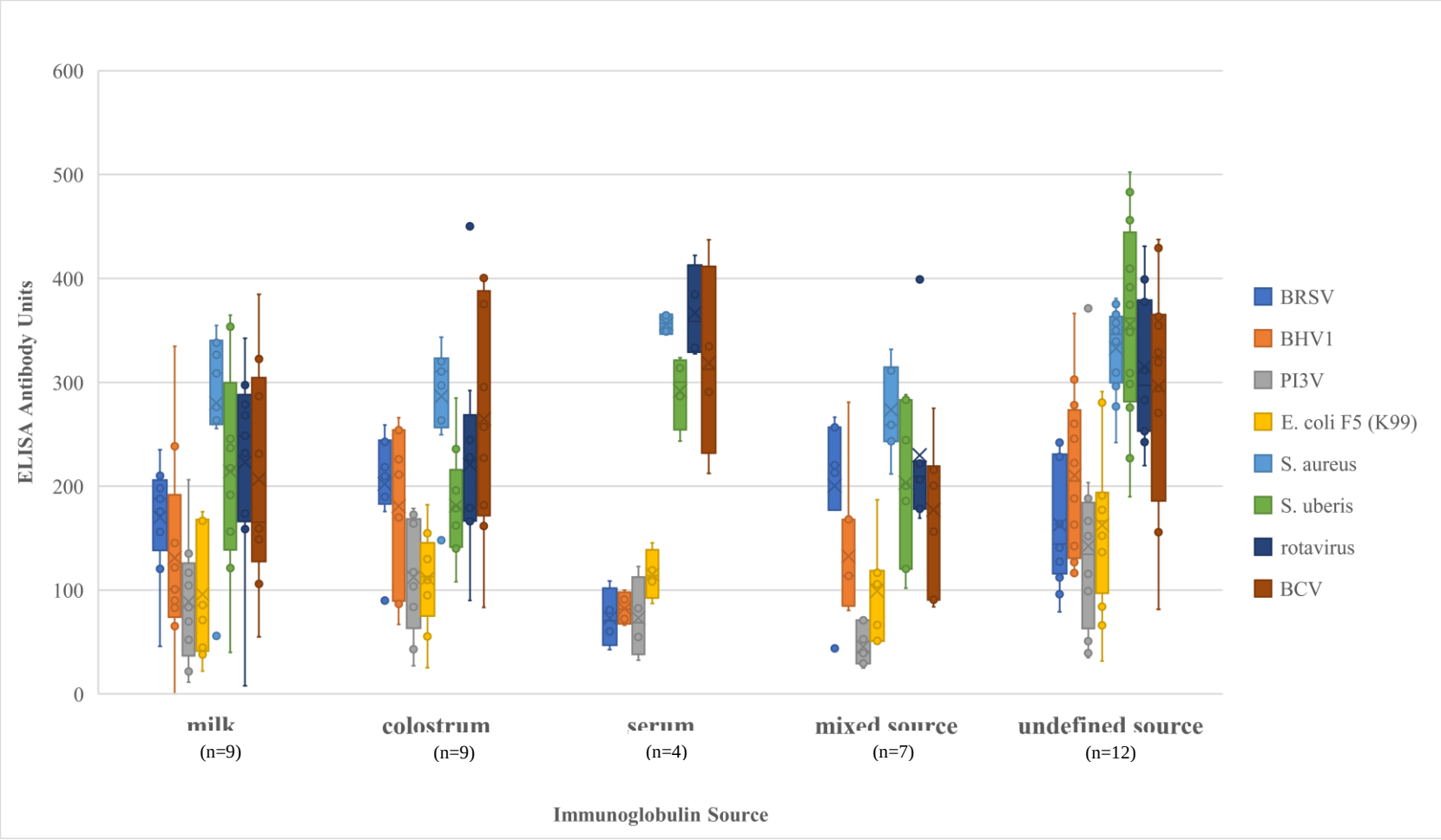
The IgG in both colostrum and milk-derived products was statistically higher than the serum IgG in specific antibodies to BRSV (Appendix B & C). Products claiming mixed sources of Igs, or for which the Ig source was unclear, tended to have variable levels of specific antibodies across the pathogen target panel used in this study, with most testing low for antibodies to BRSV, BHV-1, PI3V and *E. coli* F5 (K99).

In general, this product testing supports the findings of the animal testing in this study and indicates that colostrum-based IgG tends to have the highest coverage in terms of antibody specificities. Milk/whey-sourced IgG appears to provide comparable amounts of specific antibodies to the colostrum-derived CR formulas, and serum- based products tend to provide more specific antibody to bacterial pathogens. There were, however, differences in the serum IgG in products from tests of the individual animals IgG to some agents. It may be pertinent that post partum dams have transferred much of their serum IgG1 to the colostrum while blood-base products are from slaughter animals which may have a different profile of specific IgG.

Higher total IgG products (presumably higher quality products) are comprised of IgG with broad-spectrum antibody populations to environmentally relevant pathogens of bovid neonates, but high total IgG is not a guarantee that CR formulas will provide greater specific antibody to any one particular target, suggesting the necessity of understanding the antibody specificities of

products and their immune components. In all products, antibodies specific to the IgG of bacterial pathogens tends to be highest, which may be responsible for inflating the total IgG of lesser quality products. A better understanding of specific antibodies in CR products is one critical aspect to understanding why results of CR feed trials are variable

C.1. ELISA antibody units in the IgG of colostrum replacer products (CR). Products are grouped by immunoglobulin source. (All samples were tested at 0.5g/L total IgG and normalized to 1g/L total IgG for ELISA testing.)



C.2. Kruskal Wallis test with Dunn post-hoc analysis comparing specific antibodies in single-source commercial colostrum replacer products

ELISA Target	Kruskal-Wallis Test	Paired Treatments	Dunn Post Hoc Test
Total IgG	0.123	Serum vs. Milk	*
		Serum vs. Colostrum	*
		Milk vs. Colostrum	*
BRSV	0.010	Serum vs. Milk	0.046
		Serum vs. Colostrum	0.003
		Milk vs. Colostrum	0.191
BHV-1	0.151	Serum vs. Milk	*
		Serum vs. Colostrum	*
		Milk vs. Colostrum	*
PI3V	0.461	Serum vs. Milk	*
		Serum vs. Colostrum	*
		Milk vs. Colostrum	*
***E. coli* F5 (K99)**	0.666	Serum vs. Milk	*
		Serum vs. Colostrum	*
		Milk vs. Colostrum	*
S. aureus	0.014	Serum vs. Milk	0.716
		Serum vs. Colostrum	0.005
		Milk vs. Colostrum	0.012
S. uberis	0.050	Serum vs. Milk	0.293
		Serum vs. Colostrum	0.014
		Milk vs. Colostrum	0.105
rotavirus	0.034	Serum vs. Milk	0.637
		Serum vs. Colostrum	0.011
		Milk vs. Colostrum	0.030
BCV	0.193	Serum vs. Milk	*
		Serum vs. Colostrum	*
		Milk vs. Colostrum	*

A Kruskal-Wallis analysis was performed on ELISA ODs of single-source colostrum replacer products as a global test for differences in specific antibodies to the H+L of BRSV, BHV-1, PI3V, *E. coli* F5 (K99), *S. aureus*, *S. uberis*, rotavirus and BCV among IgG sourced from serum, milk and colostrum ($P<.05$). When differences between two biologics were significant, a post hoc analysis using the Dunn test was employed ($P<.05$).

* Represent test values that did not meet the level of significance for this statistical test. For comparisons where the Kruskal-Wallis test did not meet the level of significance, post hoc tests were not completed.